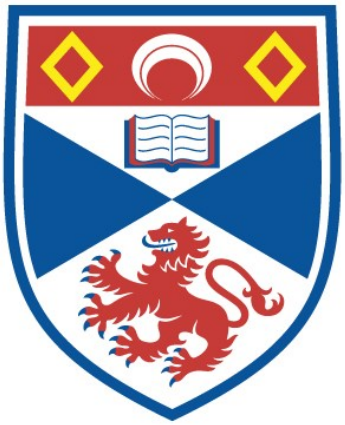
RESPONSES OF MOUSE FEMORAL BONE MARROW GRANULOCYTE-MACROPHAGE COLONY-FORMING CELLS (CM-CFC) TO X-RAYS AND RESTRICTION ENDONUCLEASES

Raja Abdul Aziz bin Raga Adnan

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1987

RESPONSES OF MOUSE FEMORAL BONE MARROW GRANULOCYTE-MACROPHAGE COLONY-FORMING CELLS (GM-CFC) TO X-RAYS AND RESTRICTION ENDONUCLEASES

A Thesis

Submitted to the University of St. Andrews for the

Degree of Master of Science in Radiation Biophysics

by

Raja Abdul Aziz bin RAJA ADNAN

Department of Anatomy and

Experimental Pathology,

University of St. Andrews,

St. Andrews, SCOTLAND.

December, 1986

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Th A 61

بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِيْمِ

ABSTRACT

The effect of X-rays on mouse normal femoral bone marrow GM-CFC was compared using two different *in vitro* cell culture techniques - the agar colony assay and the radioisotope uptake assay (^{125}IUdR or ^{3}HTdR). The D_o values obtained with the agar colony assay was 1.00 ± 0.09 Gy and 2.10 ± 0.10 Gy as measured by the radioisotope assay. Similar values were observed when the bone marrow cells were irradiated *in vitro* or *in vivo*. It was observed that the concentration of WEHI-3B conditioned medium (CSA) used in the agar colony assay would affect GM-CFC content of irradiated or normal bone marrow cells. But when optimum or greater levels of CSA were used the survival parameters plateaus.

Pre-trypsinisation of bone marrow cells was not demonstrated to have modified the radiation survival characteristics of GM-CFC to X-rays and D_o were not significantly different from GM-CFC from untreated bone marrow cells. Two distinct subpopulations of GM-CFC were observed based on their sensitivity to trypsin. One was survival-dose dependent and the other totally unaffected by increasing trypsin concentrations. Their ratio was approximately 1:1.

Trypsin permeabilised murine bone marrow cells were treated with restriction endonucleases Pvu II and Bam HI. It was postulated that these endonucleases generated blunt-ended and cohesive-ended double-strand breaks (dsb), respectively. The cells were then assayed for their clonogenic ability to simulate GM-CFC death following X-ray exposure and to test the hypothesis

that cell death arises from the induction of dsb in DNA via the formation of chromosomal aberrations . The results reported here show that Pvu II simulates X-ray exposure in causing a dose-dependent loss of the reproductive integrity of mouse femoral bone marrow GM-CFC, whilst Bam HI was found not to reduce cell survival even when concentrations greater than Pvu II were employed. These results support the idea that X-irradiated mammalian cells undergo a mode of death in which dsb in the DNA are lethal resulting in the loss of clonogenic ability. In contrast to previous experiments using inactivated Sendai virus, 0.001% trypsin was used to permeabilise the cells before treatment with the restriction endonucleases. However, when 0.05% trypsin was used Pvu II was shown not to further reduce the survival fraction of GM-CFC. The storage buffer containing the restriction endonucleases were found to be toxic but a region of tolerance was observed when the lower trypsin concentration was used. This had enabled the study of the effect of the restriction endonucleases on GM-CFC without the complicating presence of other forms of damage. From the D_{37} values, a dose of 142 $\pm$ 4 units of Pvu II was equivalent to 1.20 Gy of X-rays (ie. 100 units = 0.85 Gy).

DECLARATION

I, Raja Abdul Aziz bin RAJA ADNAN hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any degree or professional qualification.

Signed ..... Date 5-1-1987

Certified by:

Signed..... Date.....

Dr. A.C. Riches

COPYRIGHT

ACKNOWLEDGEMENTS

I would like to convey my appreciation to Professor D. Brynmor Thomas for allowing me to use the facilities within the department to carry out my research work. I would like to express my most sincere gratitude to my supervisor, Dr. A.C. Riches for his continual help and encouragement throughout the duration of this research work. I would also like to thank Dr. P. Bryant for his advice in the restriction endonuclease work. My thanks to Mr. I. Docherty, Mr. R. Stewart, Mr. J. MacIntyre, Mr. T. Smolenski, Mr. D. Ogden and Miss M. Hepburn. Special thanks to Mrs. C. Briscoe for her assistance and advice.

I would like to extend my sincere gratitude to the Department of Public Service and the Nuclear Energy Unit, The Prime Minister's Department, Malaysia, for providing the funds and the opportunity to make this thesis possible. Finally, my thanks to my family and generous friends in St. Andrews and Dundee.

ABBREVIATION AND ALTERNATIVE NAMES

A	Adenosine
ARA-C	Cytosine arabinoside
Ba-	Basophil
Bam HI	Restriction endonuclease from Bacillus amyloliquefaciens H
B-	B-lymphocyte
BFU-E	Burst colony-forming units - erythroid
BPA	Burst promoting activity
C	Cytidine
CFC	colony-forming cell
CFU-C	Colony-forming units - culture
CFU-E	Colony-forming units - erythroid
CFU-S	Colony-forming units - spleen
CM	conditioned medium
CSA/CSF	Colony stimulating activity/Colony stimulating factors
D_o/D_{37}	Dose which reduces survival to 37% when survival is exponential to dose
D_q	Point of extrapolation of the terminal slope on the linear abscissa
DNA	Deoxyribonucleic acid
dsb	double strand break
Eo-	Eosinophil
Epo	Erythropoietin
F-	Fibroblast
G	Guanosine
GM-	Granulocyte-macrophage
$^{3}_{HTdR}$	Tritiated thymidine
$^{125}_{IUdR}$	5-^{125}Iodo-2'-deoxyuridine
LD_{50}	Lethal dose to cause 50% kill
M-	Macrophage
Meg-	Megakaryocyte
n	Point of extrapolation of the terminal exponential slope on the log ordinate
Pvu II	Restiction endonuclease from Proteus vulgaris
ssb	single strand break
T	Thymidine
T-	T-lymphocyte

CONTENTS

		Page No:
1.0	INTRODUCTION	1
2.0	MATERIAL AND METHODS	31
3.0	PROCEDURES AND RESULTS	43
4.0	DISCUSSION	98
	REFERENCES	114

FIGURES

Figure No:	Title	Page No:
Figure 1.1:	The haemopoietic system	5
Figure 1.2:	Schematic diagram of the relationship of *in vivo* colony assay to *in vitro* colony assay	10
Figure 1.3:	Stimulation of the erythroid series	16
Figure 1.4:	Typical survival curves for mammalian cells exposed to radiation	21
Figure 1.5:	Target sites of Pvu II and Bam HI	29
Figure 3.1:	The number of GM-CFC colonies produced in the agar culture assay with increasing amounts of WEHI-3B conditioned medium	49
Figure 3.2:	The cell-dose response curve of the agar colony assay	51
Figure 3.3:	The effect of storage of the bone marrow cell suspension to the cell concentration and to the production of GM-CFC colonies using the agar colony assay	55
Figure 3.4:	Radioistope uptake in proliferating femoral bone cells against A) increasing concentrations of WEHI-3B conditioned medium, B) increasing number of cells plated per well and C) increasing concentrations of Foetal Calf Serum (FCS)	59

Figure 3.5A: Radiation survival curve of GM-CFC from femoral bone marrow irradiated in vivo using the agar culture assay 62

Figure 3.5B: Comparison of the survival curves of GM-CFC from femoral bone marrow irradiated in vivo using the agar culture assay and the ^{3}HTdR uptake assay 63

Figure 3.6: Radiation survival curves of GM-CFC from femoral bone marrow cells irradiated in vitro using the agar culture assay and the ^{125}IUdR uptake assay 66

Figure 3.7: Effects of CSA on the number of GM-CFC from irradiated femoral bone marrow cells 68

Figure 3.8: Effects of CSA on the percentage of the maximum number of GM-CFC from irradiated femoral bone marrow cells 69

Figure 3.9: Recovery of GM-CFC from femoral bone marrow irradiated with a split dose regimen (2 x 1 Gy) 72

Figure 3.10: Effects of different trypsin concentrations and different incubation conditions on femoral bone marrow GM-CFC survival 78

Figure 3.11: Effects of different concentrations of trypsin on the survival fraction of femoral bone marrow GM-CFC 80

Figure 3.12: Recovery of femoral bone marrow GM-CFC following trypsinisation (0.05%) 83

Figure 3.13: Response of GM-CFC from femoral bone marrow cells irradiated at different time intervals with a single dose of X-rays (2 Gy) following trypsinisation (0.05%) 87

Figure 3.14: Radiation survival curve of GM-CFC from trypsinised and untreated femoral bone marrow cells 89

Figure 3.15: Effects of endonuclease storage buffer on GM-CFC from femoral bone marrow pre-treated with A) 0.05% and B) 0.001% trypsin 91

Figure 3.16: Survival dose curve of GM-CFC from pre-trypsinised (0.05%) femoral bone marrow cells treated with A) Pvu II and B) corresponding volumes of storage buffer 93

Figure 3.17: Survival curves of GM-CFC from pre-trypsinised (0.001%) bone marrow cells treated with Pvu II restriction endonuclease and storage buffer 94

Figure 3.18: Survival curves of GM-CFC from pre-trypsinised (0.001%) bone marrow cells treated with Bam HI restriction endonuclease and storage buffer 95

Figure 3.19: Survival curves of GM-CFC from pre-trypsinised (0.001%) bone marrow cells treated with restriction endonucleases Pvu II and Bam HI compared to X-rays 96

TABLES

Table No:	Title	Page No:
Table 1.1:	The murine granulocyte-macrophage colony stimulating factors	14
Table 1.2:	Survival characteristics of mouse GM-CFC after acute doses of X- or Gamma-radiation	23
Table 3.1:	Percentage of colony types stimulated in femoral bone marrow cells using the agar culture assay with increasing CSA concentrations	48
Table 3.2:	Survival of femoral bone marrow GM-CFC on storage of cell suspension with time at different incubation temperatures ie. ice ($4^{o}C$) and in heated water-bath ($37^{o}C$)	54
Table 3.3:	Table showing the different volumes of cell suspension, Foetal Calf Serum (FCS), WEHI-3B conditioned medium and additional Fischers medium used in vials 1 - 15	57
Table 3.4:	Survival characteristics of murine femoral bone marrow GM-CFC after acute doses of X-rays	64
Table 3.5:	Colony and cluster formation in normal and X-irradiated femoral bone marrow cell cultures with increasing amount of added CSA	70

Table 3.6: Effect of different stages of the trypsinisation procedure on femoral bone marrow survival 75

Table 3.7: Recovery of GM-CFC of femoral bone marrow following trypsinisation with 0.05% trypsin (37°C, 10 mins.) 82

Table 3.8: Effect of 0.05% trypsin (37°C, 10 mins.) or ARA-C (37°C, 30 mins.) or the combination of both on the survival of femoral bone marrow GM-CFC 85

1.0 INTRODUCTION

CONTENTS

Page No:

1.1 STRUCTURE AND REGULATION OF THE HAEMOPOIETIC SYSTEM

1.1.1 Introduction 3

1.1.2 Haemopoietic stem cells 6

1.1.3 Haemopoietic progenitor cells 7

1.2 CLONAL ASSAYS FOR HAEMOPOIETIC CELLS IN VITRO

1.2.1 Historical background 8

1.2.2 Relationship to stem cells 9

1.2.3 Granulocyte-macrophage colony-forming cells (GM-CFC) 10

1.2.4 Granulocyte-macrophage colony stimulating factors (GM-CSF) 12

1.2.5 Granulopoiesis 15

1.2.6 Other methods to study proliferation activity of GM-CFC 16

1.3 X-RADIATION RESPONSES OF MAMMALIAN CELLS

1.3.1 Cell survival curves 18

1.3.2 Granulocyte-macrophage colony-forming cells 22

1.3.3 Changes in radiation sensitivity in trypsinised mammalian cells

1.3.3.1 General 24

1.3.3.2 Radiation sensitivity of mammalian cells following trypsinisation 25

1.4 RADIATION INDUCED CHROMOSOMAL ABERRATIONS

1.4.1 Introduction 26

1.4.2 Relation of chromosomal aberrations to cell death 27

1.4.3 Restriction endonucleases: Its use in the study of chromosomal aberrations caused by radiation 28

CHAPTER ONE

INTRODUCTION

1.1 STRUCTURE AND REGULATION OF THE HAEMOPOIETIC SYSTEM

1.1.1 Introduction

Haemopoiesis is an orderly chain of process by which mature blood cells are generated. It involves cell proliferation, differentiation and maturation leading to the production of functionally mature blood cells and platelets. In the adult mammal, haemopoiesis is restricted to the bone marrow, spleen, thymus and lymphoid organs (Metcalf & Moore,1971).

Haemopoietic populations are usually described as being composed of three major sub-classes of cells: stem cells, progenitor cells and a class of morphologically recognisable maturing cells. The whole population may be regarded as a three-tiered pyramidal structure with each succeeding population of a larger size than the preceeding one.

At the apex of this structure is the fundamental multipotential stem cell population with the capacity to generate the differentiated progenitor cells which may have the ability for limited self-replication. Although located mainly in the bone marrow with a small population in the spleen, a few may be found in the circulation.

As cells mature, they lose their multipotentiality and become restricted to fewer lines of differentiation and finally

to one ie. as cells progress along the differentiation-maturation pathway, more mature progenitor cells are believed to commit to one or the most two lines of differentiation. In general, only the most mature cells in each lineage (or fragments of megakaryocytic cytoplasm) enter the circulation.

Amplification of cell numbers are involved at each major stage. The late progenitor cells are limited in their capacity to proliferate and are restricted to one lineage and finally to identifiable and mature functional cells.

Until recently, it was possible to believe that two levels of control systems regulated haemopoiesis. The stem cell to progenitor cell stage was believed to be controlled by microenvironmental cells in haemopoietic tissues while humoral regulators such as erythropoietin or colony stimulating factors controlled the proliferation of progenitor cells and their progeny to form maturing cells. Recent evidence indicates that both types of control probably operate at all cell levels ie. stem cell to progenitor cell stage or control of progenitor cell proliferation (Metcalf, 1984). Regulation is generally considered to be in a dual mannner. A factor (or a family of factors) appear to act on multipotential as well as early progenitor cells in a lineage dependent-fashion. As maturation commences, the cells becomes responsive to lineage specific factors. In addition, complex cell inhibitions are believed to regulate haemopoiesis.

Presently, eight major families of haemopoietic cells are recognised to originate from the multipotential stem cells ie. erythroid, granulocytic, monocytic, eosinophilic, megakaryotic, mast cells and T- and B-lymphoid (Figure 1.1). The exact existence and origin of stem cells common to T- and B-lymphocytes

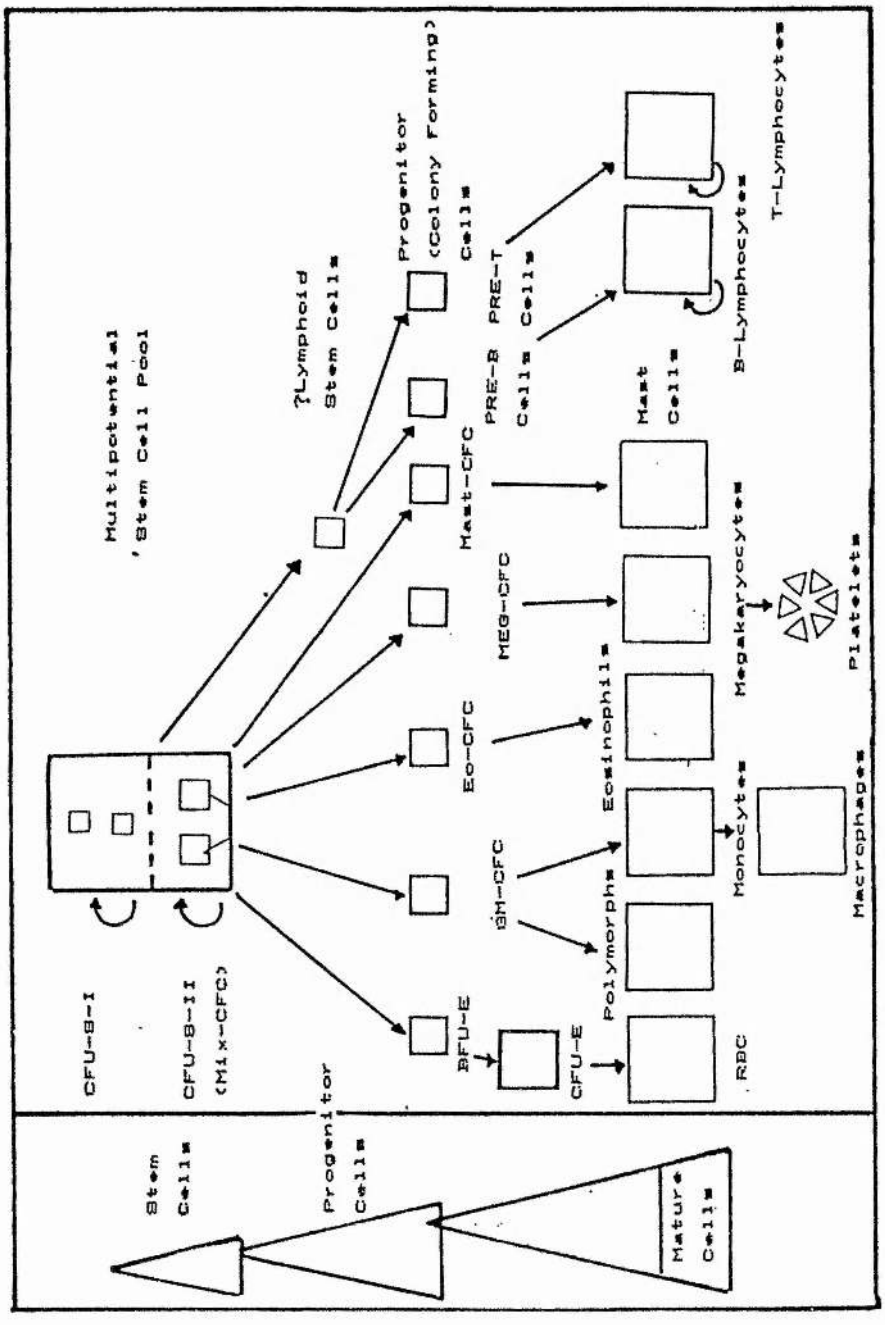


Figure 1.1: The haemopoietic system

are unresolved questions (Metcalf, 1984).

1.1.2 Haemopoietic stem cells

The earliest haemopoietic cell type has been conveniently studied experimentally as the murine CFU-S (Till & McCulloch, 1961). In this procedure, the host mice, whose haemopoietic populations are destroyed by a supralethal dose of whole-body irradiation, are injected intravenously with small numbers (0.5 - 1 x 10^5) of marrow cells. 7 - 14 days later the spleen is examined for the presence of discrete, macroscopically visible surface colonies. Analysis showed that these colonies were composed of haemopoietic cells of apparently single lineage ie. erythoid or granulocytic. At later time points colonies were composed of a mixture of various haemopoietic cells. While it was soon demonstrated that each colony was a clone derived from a single initiating cell, it was not known if whether the initiating cells required interactions with one or more associated cells in the marrow inoculum, hence the term "colony-forming unit-spleen" (CFU-S) to describe the cell(s) initiating spleen colonies.

From the study of clonal expansion of marked cells by using chromosome markers, most of the mature elements of the erythroid, lymphoid and myeloid systems were shown to be derived from a common pool of stem cells in the mouse (Schofield, 1979; Chen & Schooley, 1968; Wu et al., 1967).

It has been recognised that two distinct subsets of CFU-S are detectable by the spleen colony technique. One subset forms colonies detectable after 7 days, exhibits a relatively restricted capacity for self-regeneration and forms relatively

small populations of differentiating progeny. The second subset, detectable after 14 days more closely matches the required behaviour of repopulating cells since they have a considerably higher capacity for self renewal and generate much larger populations of differentiating progeny. It has been shown that not all the colonies visible at day 7 or 8 will be visible at days 10 or 11 or later (Magli et al., 1982)

Stem cells are relatively few in number representing 0.2% of cells in the marrow (Hendry & Lord, 1983). This fact together with that they remain morphologically unidentifiable renders them the least understood population of the tissue.

1.1.3 Haemopoeitic progenitor cells

Progenitor cells differ from stem cells in probably possesing little capacity for self replication and in being irreversibly committed to differentiation in a particular lineage. In most cases, it is a single lineage with the exeption of the granulocyte-macrophage progenitor cell population. In the mouse at least, the cells are bipotential and can generate both granulocytes and monocytes (Metcalf, 1984; Metcalf & Moore, 1973). Progenitor cells are often termed "colony-forming cells (CFC)" and preceeded by a prefix to indicate lineage eg. GM-CFC for granulocyte-macrophage colony-forming cell.

1.2 CLONAL ASSAYS FOR HAEMOPOIETIC CELLS IN VITRO

1.2.1 Historical background

The development of in vitro clonal assays for haemopoietic cells has produced a dramatic increase in our knowledge and understanding of the structure and physiology of the haemopoietic tissue. Using semi-solid culture techniques, distinct populations have been defined by their capacity to give rise to recognisable progeny.

Following the first independent reports (Bradley & Metcalf, 1966; Pluznik & Sachs, 1965) of the growth of colonies of nuetrophilic granulocytes and macrophages in soft agar, techniques for growing other classes of progenitor cells in vitro were developed. These include erthroid, megakaryocytic, eosinophilic and basophilic cell assays (as reviewed in Testa, 1985 and Metcalf, 1984). These clonal assays allow quantitative studies to be made of a whole range of haemopoietic cells, from multipotential cells not yet restricted to one lineage of differentiation, which give rise to colonies containing recognisable cells, to progenitor cells restricted to one line of differentiation and which may have limited proliferation capacity. The former are close to and perhaps overlapping with stem cells.

The proliferation of colony forming cells is dependent on the continous presence in the culture of stimulatory molecules which may be specific regulators. Thus, studies as regard to sensitivity and specificity of response as well as definition and purification of such stimulators may be examined using the in vitro clonal assays (Metcalf, 1984).

1.2.2 Relationship to stem cells

Wu et al. (1968) using a radiation-induced chromosome marker observed that similar markers were obtained in both spleen and in vitro colonies. This suggested that colonies arise from a single cell and that cells giving rise to colonies in agar are closely related to the haemopoietic cells which gave rise to spleen colonies after transplantation.

With the development of mix (or multi)-CFC assays, Metcalf and his colleagues (Johnson & Metcalf, 1977; Metcalf et al., 1979) were able to provide more data on the relationship of CFU-S and colony-forming cells. The cells forming mix colonies can contain a variety of progenitor cells and in addition multi-CFC and CFU-S. This procedure has documented the clonal nature of multipotential, erythroid, granulocytic, macrophage and granulocytic-macrophage colonies (Johnson & Metcalf, 1977). In addition, 'stem cell' colonies which do not show signs of differentiation, have the capacity to give rise to high levels of CFU-S, 'stem cell' type, mix colonies and other progenitor cell colonies (Nakahata & Ogawa, 1982).

It is not clear whether these 'stem cell' type or mix colonies assays give rise to different subsets of CFU-S (Metcalf, 1984). Figure 1.2 shows the relationship of CFU-S, 'stem cell'-CFC and mix-CFC but the tentative hierachy proposed by Testa (1985) is adopted :

CFU-S ⟷ 'stem cell'-CFC ⟶ mix-CFC

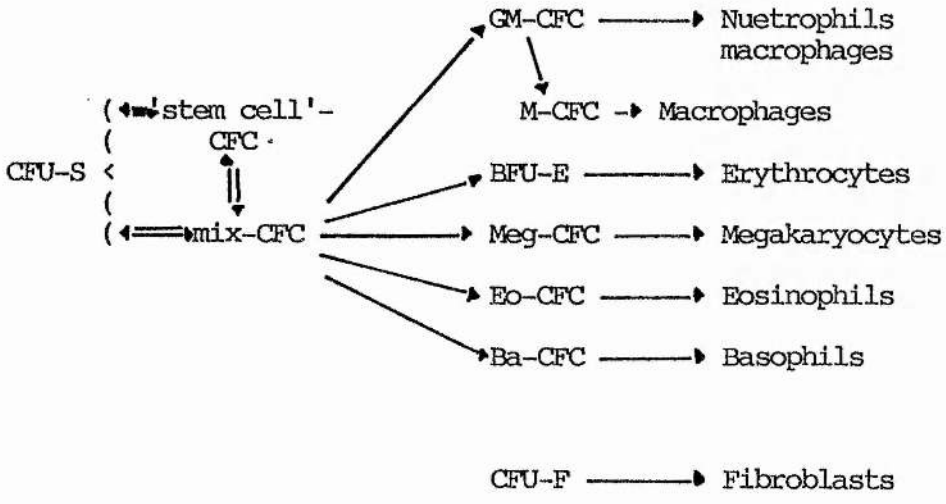
The extent of overlap between the CFU-S, 'stem cell' type and mix-CFC is difficult to establish but CFU-S have been proven experimentally to both express a more extenxive capacity for self

reproduction and giving rise to diferentiated progeny unlike 'stem cell'-CFC. The restricted capacity of mix-CFC places it behind CFU-S and 'stem cell'-CFC (Testa, 1985).

1.2.3 Granulocyte-macrophage colony-forming cells (GM-CFC)

These were the first haemopoietic cells to be grown in vitro in a clonal assay (Bradley & Metcalf, 1966 and Pluznik & Sachs, 1965). They give rise to colonies containing both granulocytes and macrophages or only one of those cell types. The assay is

Figure 1.2: Schematic diagram of the relationship of in vivo colony assay to in vitro colony assay



widely used under a variety of culture conditions. Normally murine GM-CFC are assayed either in agar, methycellulose or collagen gel. The selection of matrix depends on the aims of the experiment and its practicality. Agar is most preferred, it being easy to prepare, store and manipulate, and achieves better immobilisation of cells. However, colony formation is dependent on the final concentration of agar and 0.03% is optimal (Bradley & Metcalf, 1966). Fresh synthetic culture media is used to provide essential support for good colony growth. Buffering is normally achieved by a bicarbonate-CO_2 system in which CO_2 is supplied to the gas incubator.

Some colony assays eg. CFU-E, can be grown in the absence of serum in culture medium supplemented with bovine serum albumin, transferrin, cholesterol and soybean lipid (Iscove et al., 1980) but most assays are still performed in cultures which contain 15 - 30% serum. Historically, foetal calf serum may be the first choice however horse serum which is cheaper is used and proven to be consistently better (Dexter & Testa, 1976).

GM-CFC proliferate in response to colony stimulating factor (GM-CSF) and this may be obtained from one of the several sources (see Table 1.1).

Murine colonies are scored after 7 days of culture, but human GM-CFC may take longer. By definition a colony is 40 - 50 cells and smaller clones termed clusters are likely to outnumber colonies. These clusters are likely to be derived from the more mature population than the GM-CFC (Metcalf, 1977).

Colony size depends on a) the concentration of CSF, b) the numbers of colonies per unit volume, c) the number of cells initially plated and d) the adequacy of the media, serum, the

batch of petri-dishes and the efficiency of the CO_2 incubator in maintaining fully hydrated conditions at constant pH.

Cells from any haemopoietic organ containing the above progenitor cells can be used as the source of colony-forming cells.

1.2.4 Granulocte-macrophage colony stimulating factors (GM-CSF)

This group of factors has in common the ability to stimulate nuetrophilic granulocytes and/or macrophages to form colonies of differentiating polymorphs and/or macrophages (including eosinophils and basophils).

In Table 1.1, in the mouse, there are four major subtypes of GM-CSF with variants of similar functional properties (Metcalf, 1984 & 1986).

WEHI-3B conditioned medium is one of the two most commonly used source materials of multipotential colony stimulating factor (multi-CSF). The molecule is similar to multi-CSF synthetised by mitogen-stimulated mouse T-lymphomas or cloned T-cell lines and by certain T-lymphomas or hybridomas. It is a neuraminic acid containing glycoprotein (MW 23000). A high proportion of the colonies contain granulocytes or are composed exclusively of granulocte-containing colonies. The percentage of colonies of granulocytes (G), granulocyte-macrophage (GM)and macrophages (M) are 42, 21 and 25% respectively for C57BL bone marrow with 3200 units of purified WEHI-3B conditioned medium (Metcalf, 1984). Impure CSF will give different proportion of G, M and GM colonies depending on not only the source but also on the concentration of CSF (reviewed in Burgess & Metcalf, 1980).

In cultures containing purified multi-CSF from WEHI-3B conditioned medium, erythroid containing colonies are poorly haemoglobinised and mixed colonies contained low percentages of erythroid cells (Metcalf, 1984).

Mouse multi-CSF has no capacity to stimulate colony formation by any human progenitor cells. Other useful sources for stimulation of colony formation by mouse bone marrow cells are human urine and mouse or human serum (reviewed in Metcalf, 1984; Metcalf & Moore, 1971; and Testa, 1985).

Methods for supplying CSF to GM-CFC cultures include use of a) a feeder layer of cells (Bradley & Metcalf,1966), b) a simple system of supplying CSF to agar cultures in a small volume of fluid and c) in situ where diffusion chambers were inserted and incubated in the peritoneal cavity of irradiated mice (Gordon, 1975).

Table 1.1: The murine granulocte-macrophage colony stimulating factors

Type	Cells produced	Major sources	Molecular weight
GM-CSF	Nuerophilic granulocytes, macrophages, eosinophils (foetal).	Lung conditioned medium	23000
		Muscle conditioned medium.	23000
M-CSF	Macrophages.	L-cell conditioned medium,	70000
		Pregnant mouse uterus,	
		Yolk sac medium.	60000
G-CSF	Nuetrophilic granulocytes.	Lung conditioned medium,	25000
		Post-endotoxin serum	25000
Multi-CSF	Nuetrophilic granulocytes, macrophages, erythroid cells, eosinophils, megakaryocytes, mast cells, multipotential stem cells.	WEHI-3B conditioned medium,	23000
		Pokeweed mitogen stimulated lymphocytes	23000

1.2.5 Granulopoiesis

Using in vitro clonal culture, the colony doubling time of GM-CFC was equal to the mean cell cycle time of 14.5 hours in the first four days of culture. Starting from one colony-forming cell at the time of plating, GM-CFC completes approximately six doublings before recognisable maturing cells appear (Testa & Lord, 1973). Granulopoiesis run approximately parallel with erythropoiesis and equivalent to mylopoiesis in high demand (Hendry & Lord, 1983; Testa & Lord, 1973). The committed precursor cells constitute a population with a degree of self-replication.

The dichotomy of the GM-CFC differentiation process is not fully understood although proven that they are derived from a common ancestral cell (Metcalf & Moore, 1973).

Unlike erythropoiesis where the dual regulation model is clear (see Figure 1.3), only a similar sequence may be postulated for the granulocyte-macrophage series but is less defined and incomplete. Colony stimulating activity from purified human urine termed granulopoietin are thought to act on the committed granulopoietic precursor analogous to the action of erythropoietin on the committed erythroid precursors (Golde & Cline, 1974). However, there is no evidence it effects any differentiating step allowing the cells to proceed in vivo.

Figure 1.3: Stimulation of the erythroid series

BPA - Burst promoting activity

Epo - Erythropoietin

early BFU-E ------> CFU-E ----------> Erythrocytes

BPA Epo

Due to the diffuse distribution of the mature cells, the overall amplification of the maturation compartment is difficult to measure and are not known the number of cell cycles completed (Hendry & Lord, 1983).

At present, there is indirect evidence supporting the concept of negative feedback from the nuetrophil in the regulation of granulopoiesis but direct demonstration of this evidence for the system(s) has not been achieved (Golde & Cline, 1974).

1.2.6 Other assay methods to study proliferation activity of GMC-CFC in vitro

Radioisotope uptake (Epstein & Priesler, 1983; Twentyman et al., 1984; O'Brien & Horton, 1984) and dye exclusion (Weisenthal et al., 1983 & 1983a; Bosanquet et al., 1983) assays provide alternatives to the clonal assay of haemopoietic cell proliferation capacity stimulated by colony stimulating factors.

Dye exclusion assays are based on the ability of cells to exclude vital dyes such as eosin, nigrosin, trypan blue and erythrocin B in the presence of a physiological salt solution and

has been frequently used as an index of cell viability in vitro following exposure to cytotoxic drugs (Weisenthal et al., 1983 & 1983a).

As regards to radioisotope uptake assay, ^{3}H-Thymidine (^{3}HTdR) was initially used as to radio-label cells but it was found on dying, released their ^{3}HTdR which was then absorbed and reused by other proliferating cells (Aherne et al., 1977).

5-^{125}Iodo-2'-deoxyuridine (^{125}IUdR) has extensively been used for measurements on cell proliferation and cell loss (Dethlefsen, 1970; Dethlefsen and Riley, 1973) and for toxicity assay (Oldham et al., 1973) and problems with reutilisation is less with this marker. However, ^{125}IUdR is toxic , both chemically and radiologically. Alteration of the kinetics of the system may result if high doses were used (Aherne et al., 1977).

^{125}UdR has also the advantage of simple radiation activity measurement without extensive chemical and sample preparation compared to tritium labelled compounds.

More recently, Epstein & Priesler (1983) have developed a method for measuring the effects of chemotherapeutic agents on leukaemic cells. In this method the incorporation of ^{3}HTdR in cell DNA is used to determine the surviving portion of leukaemic cells which are stimulated to proliferate by colony stimulating factor. This method has also been used to compare the response of tumour cells to radiation and drugs (Twentyman et al., 1984). In addition, ^{3}H-Galactose has been used to monitor and manage individual patients with neutropenia (O'Brien & Horton, 1984). But in contrast to ^{125}IUdR and ^{3}HTdR which are incorporated to cell DNA, ^{3}H-Galactose incorporates into the cell membrane glycoconjugates.

1.3 X-RADIATION RESPONSES OF MAMMALIAN CELLS

1.3.1 Cell survival curves

The reproduction integrity of mammalian cells is the most important parameter to be quantitated in renewing tissues exposed to cytotoxic agents. When radiation is the cytotoxic agent, the major expression of injury is the reduction in "viability" ie. the proportion of cells which retains the ability to divide repeatedly.

These data have significant theoretical implications in both chemo- and radiotherapeutic strategy (Lajtha & Oliver, 1961; Hall, 1972; Marsh, 1976) and also in biological studies of specific tissues or cell types.

As energy from ionising radiation is deposited at random in discrete amounts, resulting generally in randomly-distributed biologial injury, it is conventional to describe and analyse "cell killing" by survival curves drawn on a semi-logarithmic coordinate system. Such a plot is primarily characterised by two parameters, n and D_o. The n describes the size of the shoulder of the curve in the low dosage range and the D_o its final slope. The radiation sensitivity of a cell population may be defined by a model which interprets such survival curves. For example, a single-hit model defines the radiation sensitivity by the dose, D_{37}, which is required to reduce the survival level from 100 to 37%.

The plot extrapolates to the origin and is truly exponential because according to Poisson distribution, when all cells have received on average one lethal hit at random, e^{-1} (= 0.37) should receive no hits. The term D_o is preferred normally where

for most mammalian cells, there is less sensitivity in the initial region ie. the shoulder and D_o is defined as the mean lethal dose describing the terminal exponential region. The size of the shoulder is described by the extrapolation number "n" which is the point of extrapolation of the terminal exponential slope on the log ordinate. Thus, comparisons of various assay techniques can be made of cell sensitivity (D_o) and of shoulder size (n). n is inversely related to the spread and the D_o is determined by the LD_{50} and the spread of the log-normal distribution (Domon, 1980).

Alternatively, the quasi-threshold dose D_q which is the point of extrapolation of the terminal slope on the linear abscissa, can be used. This value is equal to the product of (D_o . ln n) and for cells demonstrating a severe initial slope there may be a significant decrease in survival at a dose D_q. Therefore it is not representative of the true threshold dose although in a multi-target model, the linear decrease in survival per unit dose is maximal at that dose.

Besides D_o, n and D_q, survival curves may be described in other terms (eg α and β) based on different models most of which are target-type models which involves the Poisson distribution ie. "cell killing" is assumed to be lethal events occuring randomly in the irradiated cells.

The target theory of radiation action occupies a central dogma among stochastic models and appears to be the governing philosophy in radiobiology (Lea, 1955)

The repair models modify the target theory by assuming that sublethal damage is produced in cells by single hits and that the

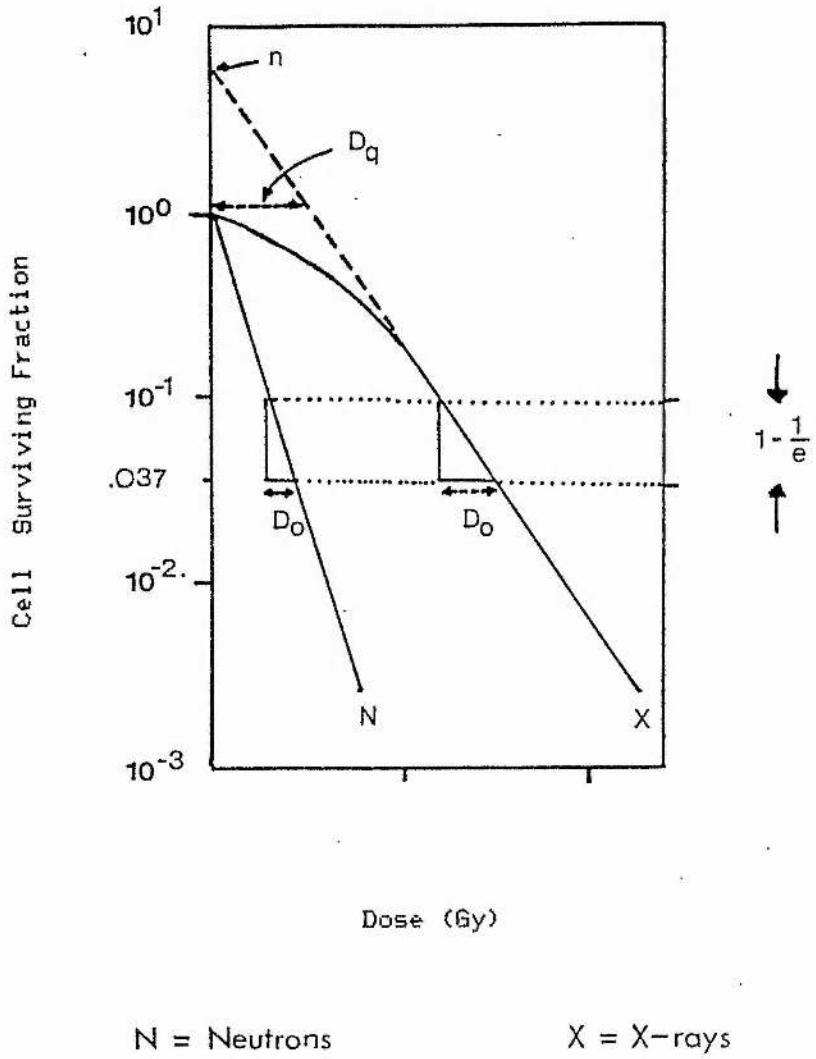
shoulder of the survival curve is due to the repair of the damage prior to its conversion to lethal damage.

The dual action model (Kellerer and Rossi, 1972; Kellerer, 1975) postulates that a survival curve is the result of a single process that has both a linear and a quadratic dependence on absorbed dose. The linear component reflecting the damage produced by a single charged particles track and the quadratic, damage arising by different charged particles.

These models are primarily concerned with a population of cells having the same biophysical parameters. When a population of cells has biological variability in their radiation response, these models require modification and interpretation. In addition, the proliferating-cell population is composed of cells in various stages of the cell-cycle (Howard & Pelc, 1953). Both cell age and oxygen concentration produce profound effects on the radiation sensitivity of cells (Tannock, 1972; Terrasima & Tolmach, 1963; Sinclair & Morton, 1966). Morever, manipulation of post-irrradiation conditions influence cell survival due to repair of potential lethal damage. Based on these observations, biological variability have been considered as a survival curve determinant (Hall, 1978).

To date, all mammalian cells studied, normal or malignant and regardless of their species of origin, exhibit X-rays survival curves similar in the form illustrated in Figure 1.4. An initial shoulder is normally present followed by a proportion which is straight or almost straight. Although the curves show some qualitative differences, their similarities is more overwhelming than their differences. With few exceptions, the 37% dose slopes (D_{37}) of the X-ray survival curves for cell lines

Figure 1.4: Typical survival curves for mammalian cells exposed to radiation. Normally, for densely ionising radiations eg. low energy neutrons, the dose-response curve is a straight line. For sparsely ionising radiations eg. X-rays, the dose-response curve has an initial shoulder followed by a portion which is straight.



N = Neutrons X = X-rays

cultured in vitro fall between 1.0 and 2.0 log and the extrapolation numbers appear to be more variable (Hall, 1978).

Survival curves for clonogenic cells in normal tissues exhibits a generally higher extrapolation number when assessed in vivo than in vitro which may be partly due to lack of detection of repair of potentially lethal damage in in vitro assays (Hendry, 1985).

1.3.2 Granulocyte-macrophage colony-forming cells (GM-CFC)

In the case of haemopoietic cell survival curves, extrapolation numbers vary between 0.9 to 1.5 (Hendry, 1985). The most radiosensitive cells in the haemopoietic cell hierachy are the stem cells and the erythroid progenitors and both have a similar sensitivity with D_o (X-rays) = 0.74 Gy and n = 1.8. Stem cell killing is not related to their self renewal capacity (Hendry & Lord, 1983). GM-CFC is apparently the most resistant D_o (X-rays) = 1.8 Gy, n = 0.94 in the mouse. Generally, mouse GM-CFC is similar in their sensitivities to human GM-CFC and having no shoulder (Hendry & Lord, 1983; Hendry, 1985). Table 1.2 shows the survival characteristics of mouse GM-CFC after acute doses.

Senn & McCulloch (1970) presented the first survival curves measured immediately after single aute doses of ^{137}Cs-Gamma rays for mouse and human GM-CFC and subsequently followed by other data on mouse, human and dog GM-CFC (reviewed in Hendry & Lord, 1983). The radio-sensitivity of pre-GM-CFC using the diffusion chamber assay is generally similar to that measured using the in vitro clonal assays. The advantage of the diffusion chamber technique lies in its independence of CSA required for colony

Table 1.2: Survival characteristics of mouse GM-CFC after acute doses of X- or Gamma-radiation. Alphabets in brackets denotes reference(s)

Radiation	Dose rate	Source of cells	Irradiation conditions	D_o (± SE)	Extrapolation number n (± SE)	
^{60}Co	1.03 Gy min^{-1}	LACA femoral marrow	in vivo	1.737	0.92	(a)
	0.032 Gy hr^{-1}			0.721	1.05	
^{60}Co	0.55 Gy min^{-1}	C57/BL femoral marrow	in vivo	2.01 (0.14)	0.51	(b)
?	?	C57/BL	?	1.97	-	(c)
X-rays 180 kVp 20 mA	2.5 Gy min^{-1}	ddY bone marrow	in vivo	1.55	1	(d)
^{137}Cs	1.12 Gy min^{-1}	C3H/He	in vivo	1.35 (0.1)	1.10 (0.06)	(e)
			in vitro	1.24 (0.08)	1.25 (0.24)	
Estimated Mean of 4 measurements				1.79 (0.10)	0.94 (0.05)	(f)
			Mean	1.61 (0.11)	0.96 (0.05)	

(a) Wu et al., 1983.
(b) Millard & Blackett, 1981.
(c) cited in Gordon, 1975.
(d) Aizawa et al., 1984.
(e) Lin, 1975.
(f) Hendry & Lord, 1983 (estimated from Senn & McCulloch, 1970; Testa et al., 1973; Wilson et al., 1974; and Wu et al., 1978).

growth in vitro. Measurements using granulocytic population ability gave a D_o value of 1 ± 0.14 Gy X-rays (Hellman et al., 1970).

GM-CFC sensitivity is influenced by levels of CSA and therefore affects D_o values (Broxymeyer et al., 1976; Sugavara et al., 1980). There is a lack of dose-rate for the cycling GM-CFC (Testa et al., 1973) although there is also evidence that the difference in the survival response in acute and chronic irradiation is very marked (Wu et al., 1983).

1.3.3 Changes in radiation sensitivity in trypsinised mammalian cells

1.3.3.1 General

Preparation of single-cell suspension of established cell lines ie. those which will proliferate indefinitely in vitro of animal or human sources, are often normally detached by trypsinisation with the minimum possible concentration of trypsin in buffered saline (0.05 - 0.25% trypsin) (Fox, 1986).

Mammalian cell permeabilisation procedures using trypsin as the permeabilising agent in experiments involving restriction endonucleases treatments has not been extensively reported to date (Obe et al., 1985; 1986). It use is of considerable importance so as to provide a cheaper and non-viral alternative to inactivated Sendai virus.

Trypsin cleaves proteins between specific amino acid residues and cut on the carboxyl side of lysine or arginine residues. Since it cleaves at relatively few sites in a protein, they tend to produce large peptides (Alberts et al., 1983). Therefore would to a limit, retain the integrity of a cell

membrane.

1.3.3.2 Radiation sensitivity of mammalian cells following trypsinisation

Several reports (Barendsen & Walter, 1964; Philips & Tolmach, 1964; Berry et al., 1966; Lehnert, 1975) have reported that a decrease in radiation sensitivity of mammalian cells exists as the time between trypsinisation, plating and irradiation is increased. These reports in particular, have suggested that mammalian cells have become considerably more resistant in split dose experiments and attributed the cause to the action of trypsin on the cells. Results from split dose experiments (Bryant & Parker, 1979) show that the increase in resistance to second doses of X-rays can be accounted for by the post-trypsinisation increase in survival and not as a result of the previous dose of X-rays. Philips & Tolmach (1964) reported that trypsin causes cells to be partially synchronised and this was evident by a transient suppression of the incorporation of the ^{3}H-Thymidine into DNA. Further, split dose experiments show an increase in survival followed by a decrease with time after trypsinisation. The peak in resistance occurring at the time of maximum DNA synthesis reflects the progression of cell cohorts through the cell cycle. Berry et al. (1966) using Chinese hamster and HeLa cells also concluded that increase in survival occurs after trypsinisation and also suggest the cause of the phenomena to synchronisation and accumulation of cells in a more resistant phase of the cell cycle.

1.4 RADIATION INDUCED CHROMOSOMAL ABERRATIONS

1.4.1 Introduction

Radiation induced chromosomal aberrations that are observed at cell division are a result of two processes - radiation breakage either of the chromosome or of the chromatid, followed by the rejoining of the broken ends in various rearrangements. The aberrations may be divided into one-hit or two hit types. The frequency of simple one-hit breaks is approximately linearly related to dose and independent of dose rate. The more complex two hit breaks increase in frequency more rapidly than the single power of the dose and the yield is related to the rate at which radiation is delivered (Lloyd et al., 1975). Two hit aberrations show a split dose effect, the rejoining (ie.repair) of the breaks induced by the first dose occuring before the second dose is received causes a reduced yield of two-hit aberrations relative to the yield caused by a single dose of the same magnitude (Coggle, 1977).

The nature of the rejoining phenomenon have shown it to be an active process, requiring oxidative phosphorylation and protein synthesis but the exact nature is unknown and must await detailed knowledge of the chromosome structure (Coggle, 1977).

Present knowledge allows only speculations as to how chromosome breaks can cause cell death. The induction of dominant lethal mutations due to damage at the initial site of a break as a result misrepair may cause cell death. So too does the loss of certain pieces of information carried when for example acentric fragments are formed leading to lethal consequences for one or both of the daughter cells, or anaphase bridges may snap so that

unequal distribution of chromosonal material between the daughter cells result in death in one or both daughter cells. Anaphase bridge formation may prevent the physical separation of the daughter cells (Carrano, 1973).

1.4.2 Relation of chromosomal aberrations to cell death

When mammalian cells are exposed to x-rays loss of normal colony forming ability is nearly always preceded by the loss of genetic material in the form of chromosome fragments or micronuclei arising from chromosonal aberrations (Grote & Revell, 1972; Joshi et al., 1982). Conversely normal growth developed where no fragment loss had been observed.

It may also be likely that on asymmetrical chromosome exchange (dicentric, centric ring, or tricentric) are capable of causing cell death where translocation and inversions does not (Carrano, 1973). The initial decrease in viability is predominantly caused by the formation of anaphase bridges and fragment loss increasingly important in later generations.

Chromatid abberrations may or may not be lethal depending on whether only one or both chromatids are affected (Dewey et al. 1971).

The particular lesion or lesions in the chromosomes responsible for loss clonogenic ability is not clearly defined as there may be lesions in chromosomes undetectable at cytological level which do cause and probably detectable aberrations such as chromatid exchanges which do not (Dewey et al., 1971). Strong evidence exists for the close parallelism between chromosomal aberration induced and loss of reproduction integrity following

X-ray exposure (Dewey et al., 1971; Carrano, 1973).

The study of a resistant cell line compared with its sensitive parent eg. mouse lymphoma L5178YR and S lines suggests that the effects are causally related or at the least they have a common origin in the form of the same type of primary lesion in DNA (Scott, 1983). Hence it is likely that a DNA lesion is responsible for both loss of proliferative capacity and chromosome aberrations may arise from a double strand break (dsb). Data obtained from conditionally repair-deficient strain of yeast confirms that dsb in DNA are the cause of cell death after irradiation (Frankenberg et al., 1984). Increase in the chromosome aberration yield have been shown to result when X-irradiated and permeabilised mammalian cells were treated with single strand specific nucleases (Natarajan et al., 1980). Single strand breaks induced by the X-irradiation procedure are believed to be converted to dsb by the addition of these specific nucleases.

1.4.3 Restriction endonucleases: Its use in the study of chromosomal aberrations caused by radiation

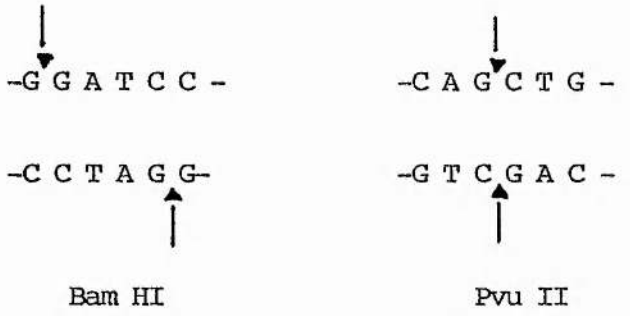
It has been recently shown that the induction of dsb in the DNA by restriction endonucleases of mammalian cells permeabilised by inactivated Sendai virus caused the formation of chromosomal aberrations (Bryant, 1984; Natarajan & Obe, 1984). This evidence has been translated into loss of clonogenic ability due to formation of chromasomal aberrations caused by the treatment of restriction endonucleases (Bryant, 1985).

Restriction endonucleases are endo-deoxyribonuclease that digest double stranded DNA after recognising specific nucleotide

sequences by cleaving two phosphodiester bonds, one within the double stranded DNA. However there are fundamental differences between several classes of restriction enzymes (termed class 1, 11 and 111) that distinguished by their specifity of their cleavage and modification reaction and their dependence upon various cofactors.

In contrast to restriction endonucleases of class 1 or 111, the enzymes Pvu II and Bam HI belonging to class 11, are characterised not only by specific DNA recognition but also by cleavage DNA at fixed positions (Figure 1.6). The catalytic functions of class 11 enzymes require Mg^{2+} as the sole essential factor. The two phosphodiester bonds attacked by the enzymes are found within or nearby the recognition sequences of tetra-, penta-, hexa-, or hepta-nucleotides. Hydrolysis results in fragments with blunt ends (Pvu II) or cohesive ends (Bam HI).

Figure 1.5 Target sites of Pvu II and Bam HI



G - guanosine A - adenosine T - thymidine C - cytidine

Although both ends are 5'-phosphorylate and 3'-hydroxylated fragments, "cohesive" or "sticky" fragments are single stranded 5'- or 3'- protruding ends of variable length and can associate by hydrogen bonding between overlapping 5'-termini or the

fragments can circularised by intramolecular reaction (Old & Primrose, 1985).

In general, the oligonucleotides sequences recognised by restriction enzymes of class 11 have a two fold axis of symmetry taking into account the polarity of two DNA-chains.

The incubation conditions that alter specificity may be produced by reducing ionic strenght, increasing the pH, substitution of Mn^{2+}, Co^{2+} or Zn^{2+} for Mg^{2+}, raising the enzyme concentrations, using high concentrations of glycerol (> 20%) or by addition of organic solvents (Boehringer Manhiem, 1985).

In this thesis, the effects of X-irradiation on murine femoral bone GM-CFC will be studied using the agar culture assay and the radioisotpe uptake assay. Responses of the cells to X-irradiation in vivo and in vitro will be compared. The effect of trypsin and its role as a radiation response modifier on GM-CFC will also be investigated.

To simulate X-ray damage, restriction endonucleases Pvu 11 and Bam H1 will be tested for their potential killing of GM-CFC in femoral bone marrow cells permeabilized with trypsin.

2.0 MATERIALS AND METHODS

CONTENTS

Page No:

2.1 EXPERIMENTAL ANIMALS 33

2.2 IRRADIATION TREATMENT

2.2.1 In vivo irradiation 34

2.2.2 In vitro irradiation 34

2.2.3 Split dose regimen 34

2.3 PREPARATION OF BONE MARROW SUSPENSION 35

2.4 CELL COUNTING 36

2.5 IN VITRO CULTURE OF GRANULOCYTE-MACROPHAGE PROGENITOR CELLS

2.5.1 Agar culture assay 36

2.5.2 Radioisotope uptake assay 37

2.6 TRYPSINISATION OF CELLS

2.6.1 Trypsin-EDTA buffer 39

2.6.2 Cell permeabilisation with trypsin 40

2.7 RESTRICTION ENDONUCLEASE TREATMENT 40

2.8 SUICIDE TECHNIQUE USING CYTOSINE ARABINOSIDE (ARA-C) 41

2.9 STATISTICAL ANALYSES 42

CHAPTER TWO

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Male and female CD1 mice were used in all the experiments. The CD1 mice originated from and were supplied by Charles River (U.K) and outbred in the University of St. Andrews Animal House.

The mice stocks were kept in a room with a lighting cycle of 12 hours light and 12 hours darkness at a temperature of 22^{o}C. The mice were fed with R & M Diet No:1 pellets (B.P., Witham, Essex) and water containing 15 ppm of free chloride ion (Kirbychlor, Mildenhall, Suffolk).

Mice used in the experiments were of ages between six to eight weeks. During an experiment, the live mice were kept in presterilised cages lined with clean saw-dust in groups of five.

2.2 IRRADIATION TREATMENT

Irradiation treatment was performed by using a Siemen Stabilipan X-ray machine (250 KVp, 14 mA, and 0.5 mm Cu filtration) fixed with an applicator which gave a 20 x 20 cm^{2} of radiation field. The dose rate was measured with a Farmer dosimeter type 2502/3 (Nuclear Enterprises Ltd, U.K.) with a thimble-type ionisation chamber. The cell suspension was irradiated about 1 hour after preparation.

2.2.1 In vitro irradiation

The chamber was placed in a plastic round-bottom tube (Sterilin, U.K.) whiched was fixed in the centre of the radiation field. The mean dose rate was 0.7512 $\pm$ 0.0052 Gy min^{-1}. The volume of cell suspension irradiated was kept constant at 3.0 ml.

2.2.2 In vivo irradiation

The chamber was placed in the centre of a wax, tissue-equivalent mouse phantom and the mean dose rate at different positions in the perspex irradiation cage was taken. At 55.5 cm focal skin dose the dose rate was 0.88 Gy min^{-1}. Mice were irradiated in groups of three in a perspex irradiation cage, with individual radially disposed compartments.

2.2.3 Split dose regiment

Each cell suspension was irradiated with two equal doses of X-radiation, D_1 and D_2. Within an experiment, all the test suspensions were irradiated with an equal total dose of irradiation. Unless otherwise stated, the cell suspensions were kept on ice before the initial dose and in the 37°C heated water-bath during the duration between the two irradiation doses, T. The initial dose, D_1 was given at time, $t = -T$ (where T is the duration between the two equal doses of X-rays). When $t = 0$, the final dose, D_2 was given simultaneously to all the test cell suspensions, after which they were kept on ice until plated for assay.

2.3 PREPARATION OF BONE MARROW CELL SUSPENSION

The mice were sacrificed by means of cervical dislocation and if the mice were irradiated *in vivo*, they were sacrificed within 1 hour after irradiation treatment. The skin was swabbed with 70% alcohol before dissecting the tissues out. The femur was dissected free of muscle and rapidly removed by cutting above the hip joint with scissors and disarticulated at the knee joint with the aid of a scalpel blade. The adherent muscles and adhering connective tissues were stripped off the femur shaft and the femur cut below the lesser trochanter. The femurs were then placed in a sterile 35 mm petri dish (Sterilin Ltd., Middlesex) containing cold medium supplemented with 50 i.u. ml^{-1} penicillin (Glaxo, England), 50 mg ml^{-1} streptomycin (Evans, England), 2 mM glutamine (Flow Laboratories, Scotland) and either with or without 25% Horse Serum (HS; Gibco Ltd., Scotland). The marrow cells were then removed by inserting a 23G x $^{1}/_{4}$ inch needle into the inter condylar notch and flushed with 1 ml of either partially or fully supplemented medium through the bone cavity by using a 1 ml Tuberculin syringe (Plastipak, Ireland) into a plastic or glass container. To produce a single cell suspension the marrow plugs disharged were further dispersed through repeated aspirations using a 25G x $^{5}/_{8}$ inch needle (Becton, Dickinson & Co. Ltd, Ireland). The suspension was then diluted to the desired cellularity.

2.4 CELL COUNTING

Bone marrow cell suspensions were counted using a Coulter Model D (Industrial) Counter (Coulter Electronics Ltd., England). The counter settings were fixed at plateau phase, optimum for the counting of granulocytic cells of murine origin. Cells in suspension were counted by diluting 100 µl into 20 ml of Isoton (Coulter Electronics Ltd., England) and 3 - 5 drops of Zaponin (Coulter Electronics Ltd., England) were added to lyse the erythrocytes.

2.5 IN VITRO CULTURE OF GRANULOCYTE-MACROPHAGE PROGENITOR CELLS (GM-CFC)

2.5.1 Agar Culture Assay

Granulocyte-macrophage colony forming cells (GM-CFC) were assayed in semi-solid agar in the presence of colony stimulating activity (CSA) obtained from the mouse myelomonocytic leukaemia WEHI-3B cell line (Pattersons Laboratories, Manchester) conditioned media.

Bone marrow cells were suspended in Dulbecco's Modified Eagle's medium (Flow Laboratories, Scotland) supplemented with 25% horse serum, 2mM glutamine, 50 i.u. ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. The cell concentration was adjusted to a concentration which will produce 20 - 150 colonies when finally plated. 3% of Bactoagar (Difco Laboratories, Surrey) was placed in a boiling water-bath for about 10 minutes before being used in the assay procedure. 1 part by volume of boiled agar was added into 8 parts by volume of Dulbecco's fully supplemented medium

and lightly swirled in a 25 ml glass universal bottle. This was then followed by the addition of 1 part by volume of bone marrow cell suspension. The mixture was then thoroughly mixed and aliquots of 1 ml were rapidly dispensed into 35 mm petri dishes (Sterilin Ltd., Middlesex) containing 100 µl of WEHI-3B conditioned media. The cultures were allowed to gel for about 10 minutes and incubated at 37°C in humidified atmosphere containing 5% CO^2 in air. Colonies containing more than 50 cells were scored after 7 days of incubation by using an inverted microscope (Kyowa, Tokyo) at x 40 magnification.

CSA controls ie cell suspensions plated without any CSA were included in all assays involving this agar culture system. These acted as controls for the production of endogenous CSA in the test assay.

2.5.2 Radioisotope Uptake Assay

Granulocytic cells actively synthesising DNA were assayed in the presence of colony stimulating factor (CSF) obtained from the mouse myelomonocytic leukaemia WEHI-10 cell line conditioned media and ^{125}IUDR (5-^{125}Iodo-2'-deoxyuridine). The proliferative response was determined by the uptake of the ^{125}IUDR, a radioisotope labelled DNA precursor, as a measure of proliferation

Bone marrow cells were suspended in RPMI 1640 (Flow Lab., U.K.) medium supplemented with 2 mM glutamine, 50 i.u. ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. The cell suspension was diluted to a cell concentration of approximately 1 x 10^7 cells ml^{-1}. Between 1 - 3 x 10^6 cells were aliquoted to 5 ml glass

vials and to it was added 200 /ul WEHI-3B (10%), 200 /ul FCS (10%). The mixture was then made up to 2 ml with RPMI 1640 supplemented with penicillin/streptomycin and glutamine. Controls were treated with 10% of the same RPMI 1640 supplemented medium. 200 /ul of WEHI-treated and control cells were then aliquoted to microtitre wells (Nunclon, InterMed, Denmark). The microtitre plates were then incubated for 2 days at 37°C in a humidified atmosphere containing 5% CO_2 in air, after which 20 /ul (approximately 0.2 /uCi = 7.4 kBq) of ^{125}IUDR (approximately 5 Ci = 185 GBq mg^{-1} at activity reference date; Amersham International Ltd., Amersham, Bucks.) was added to each well and incubation continued for another day. At the end of the incubation period, the cells were then harvested using a Titertek Cell Harvester (Flow Laboratories Ltd., Irvine, Ayrshire). Cells were drawn and washed with normal saline (0.9% NaCl) onto a paper filter. These filter papers were then dried by placing them into an oven having a temperature of 45oC and left to dry overnight.

Dry filter discs were placed in plastic counting vials (LKB Instruments Ltd., Croydon) and counted using a Mini-Gamma Counter Model 1275 (LKB, Wallac, Finland). Counts were normalised to the activity of the initial disc having taken into account background radiation. ^{125}IUDR was stored at 4°C in a refrigerator to maintain stability.

Tritiated thymidine (^{3}HTdR) was also used in this procedure instead of ^{125}IUDR. 25 /ul of ^{3}HTdR (18 /uCi = 666 kBq ml^{-1} at activity reference date; Amersham International Ltd., Amersham, Bucks.) was added to the plates in the same manner as ^{125}IUDR was added. The resultant dry filter discs were placed in plastic scintillation vials (LKB Instruments Ltd., Croydon) and to it was

added 2 ml of scintillant (Micellar Scintillator NE, Nuclear Enterprises Ltd., Edinburgh). These vials were then placed in the fridge ($4^{o}C$) for 12 hours and then counted in a scintillation counter Model 1214 RACKBETA (LKB, Wallac, U.K.) for ^{3}H activity. Results were treated similarly as when ^{125}IUDR was used.

2.6 TRYPSINISATION OF CELLS

2.6.1 Preparation of Trypsin-EDTA buffer

PBS-EDTA buffer was prepared by dissolving the following salts (Sigma cell culture reagents, U.S.A) to 1 litre of double-distilled water.

Sodium chloride, NaCl	8.0 g
Potassium chloride, KCl	0.2 g
Potassium hydrogen chloride, Na_2HPO_4	1.15 g
Potassium hydrogen sulphate, KH_2PO_4	0.2 g
EDTA	0.2 g

The solution was then sterilised by passing through millipore filters and dispensed in 100 ml aliquots. Dry trypsin powder (Bactrotrypsin, Difco Laboratories, Surrey, U.K.) was then dissolved in double-distilled water and diluted to a concentration of 0.5%. This trypsin solution was then dispensed into 1 ml aliquots. Both the PBS-EDTA buffer and the trypsin solution were stored in deep freeze.

A working solution of 0.05% trypsin was obtained by adding the 1 ml aliquot of the 0.5% trypsin to 100 ml aliquot of the PBS-EDTA buffer.

2.6.2 Cell permeabilisation with trypsin

Normal bone marrow cells were permeabilised with Bactotrypsin (Difco Laboratories, Surrey, U.K.) and the effect of trypsin stopped by simple saturation with sufficient amount of protein in the form of horse serum.

Normal bone marrow were suspended in protein-free Fischer's medium (Flow Labs., U.K.) supplemented with 2 mM glutamine, 50 i.u. ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. Between 3 - 5 x 10^6 cells were spun at 1000 rpm for 15 minutes using a refrigerated centrifuge Model Chilspin 2 (MSE, England) and the supernatant removed. 2 mls of cold trypsin solution of known concentration was added and thoroughly mixed, followed by a period of incubation at 37°C in a water-bath for 10 minutes. At the end of incubation, 20 /ul of horse serum was added. The suspension was then centrifuged at 1000 rpm for a further 15 minutes. The supernatant was again removed and to the remaining pellet of cells was again added 20 /ul of horse serum. This was to curtail any residual activity of trypsin. Test samples were kept on ice before being assayed or for further response treatments.

2.7 RESTRICTION ENDONUCLEASE TREATMENT

The restriction endonucleases were added to the trypsin-permeabilised normal bone marrow cells and incubated. The reaction was halted by diluting the reaction mixture by adding medium approximately 100x the volume of reaction mixture and placing the final suspension on ice.

Appropriate amounts of restriction endonucleases PVu II or Bam H1 (Amersham International plc., Amersham, Bucks.) were added to the trypsin-treated bone marrow cells and mixed thoroughly. A period of incubation follows at 37°C in a water-bath for 30 minutes. At the end of incubation, additional Fischer's medium was added and the concentration adjusted to produce 20 - 150 colonies when plated. The suspensions were then placed on ice before being assayed.

Trypsin controls (ie. trypsinisation only) and storage buffer controls (ie. corresponding volume of storage buffer containing no restriction endonuclease) were included in the response experiments.

2.9 SUICIDE TECHNIQUE USING CYTOSINE ARABINOSIDE (ARA-C)

The percentage of the colony forming units undergoing DNA synthesis ie. in S-phase was measured by using the following suicide technique. An S-phase specific drug Cytosine Arabinoside (ARA-C; Upjohn, U.K) was used (Cork et al., 1981).

Normal bone marrow were suspended in protein-free Fischer's medium supplemented with 2 mM glutamine, 50 i.u. ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. The cell concentration was adjusted to 2×10^6 - 2×10^7 cells per ml. To a volume of cell suspension was added an equal amount of ARA-C (40 $\mu g\ ml^{-1}$) giving a working concentration of ARA-C of 20 $\mu g\ ml^{-1}$. A same volume of medium was added to the control. The cell suspensions were then incubated in a heated water bath at 37°C for 60 minutes. At the end of incubation the cell suspensions were washed three times.

This involves spinning the cells at 1000 rpm for 15 minutes and resuspending in fresh medium and this procedure was repeated for the stated number of times. Prior to plating, the cell suspension was gently aspirated using a syringe and needle as described earlier. The cellularity was determined and diluted for assay of GM-CFC using the agar culture assay.

2.9 STATISTICAL ANALYSES

Linear regression analysis, linear correlation studies, two-way or one-way analysis of variance (F-test) and Students T-test were performed using the SUPERSTATS™ program (Lombardy Scientific Ltd., U.S.A.), a statistical package available on the APPLE II personal computer (64K). The values of $D_{o/37}$ and n were also determined using this computer program package.

The comparison of regression coefficients for two independent sets of data were performed using the two-step method detailed in Parker, 1986 (pp.96). Only if the difference is non-significant in the preliminary test is the comparison of regression coefficients valid.

From the tests of significance, the level of probability and points of significance were determined using the SUPERSTATS™ program and/or the Cambridge Mathematical and Statistical Tables (Powell, 1976; pp. 56-59).

3.0 PROCEDURES AND RESULTS

CONTENTS

Page No:

3.1 PRELIMINARY EXPERIMENTS

3.1.1 Agar culture assay

3.1.1.1 Response of GM-CFC of normal murine femoral bone marrow cells to varying concentrations of WEHI-3B conditioned medium 46

3.1.1.2 Determination of the relationship between number of normal femoral bone marrow cells plated to the total number of colonies produced 50

3.1.1.3 Effect of pre-incubation of normal femoral bone marrow cell suspension on the total number of colonies produced 52

3.1.2 Radioisotope uptake assay

3.1.2.1 Effect of different incubation conditions on the uptake of 5'-^{125}Iodo-2'-deoxyuridine (^{125}IUdR) by proliferating normal bone marrow cells 56

3.2 RADIATION RESPONSES OF NORMAL BONE MARROW GRANULOCYTE-MACROPHAGE PROGENITOR CELLS

3.2.1 Radiation responses of GM-CFC in femoral bone marrow of mice irradiated in vivo (whole-body irradiation) 60

3.2.2 Radiation responses of GM-CFC from the femoral bone marrow of mice irradiated in vitro 65

3.2.3 Effect of concentration of colony stimulating activity (CSA) on the number of GM-CFC of irradiated femoral bone marrow 67

3.2.4 Response of femoral bone marrow GM-CFC to a split-dose of X-rays 71

3.3 TRYPSINISATION EXPERIMENTS

3.3.1 The effects of procedures involved in the trypsinisation process on femoral bone marrow GM-CFC survival 73

3.3.2 The effect on femoral bone marrow GM-CFC survival following different incubation conditions during trypsinisation 76

3.3.3 The effect of increasing concentrations of trypsin on femoral bone marrow GM-CFC survival 79

3.3.4 Recovery of femoral bone marrow GM-CFC following trypsinisation 81

3.3.5 Determination of the proportion of trypsin-sensitive femoral bone marrow GM-CFC in DNA synthesis (ie. in S-phase) 84

3.3.6 The response of femoral bone marrow GM-CFC to a single dose of X-rays administered at various intervals following trypsinisation 86

3.3.7 Radiation responses of murine femoral bone marrow GM-CFC following trypsinisation 88

3.4 RESTRICTION ENZYME EXPERIMENTS

3.4.1 The survival response of pre-trypsinised femoral bone marrow GM-CFC to restriction endonucleases (Pvu II and Bam HI) and endonuclease storage buffer 90

CHAPTER THREE

PROCEDURES AND RESULTS

3.1 PRELIMINARY EXPERIMENTS

3.1.1 Agar culture assay

3.1.1.1 Response of GM-CFC of normal murine femoral bone marrow cells to varying concentrations of WEHI-3B conditioned medium.

Experimental procedure

A normal bone marrow cell suspension was prepared in a fully supplemented Dulbecco's medium (ie. with 25% horse serum) and the cell concentration was adjusted to 8.5×10^5 cells per ml. The cells were plated normally using the GM-CFC agar culture assay. The petri-dishes into which the cells were plated were divided into five groups of three containing WEHI-3B conditioned medium in final concentrations of 0 (CSA control), 4.8, 9.1, 13.0, and 16.7% per plate. These corresponded to the addition of WEHI-3B conditioned medium of either 0, 50, 100, 150 and 200 µl per plate respectively.

Results

No colonies were present in the controls ie. those plates without any CSA. But those plated with WEHI-3B conditioned medium gave rise to colonies containing both granulocytes and macrophages or only one of those cell types. However different concentrations of WEHI-3B conditioned medium gave different proportions of granulocyte-macrophage (mix), macrophage (loose),

and granulocyte (tight) colonies significant at $P < 0.1$ (F-test; Table 3.1).

From Figure 3.1, it is evident that with increasing volumes of CSA added to the assay plates, the total number of colonies produced shows a rapid initial rise which plateaus when the concentration of added CSA was approximately 7.0% and onwards.

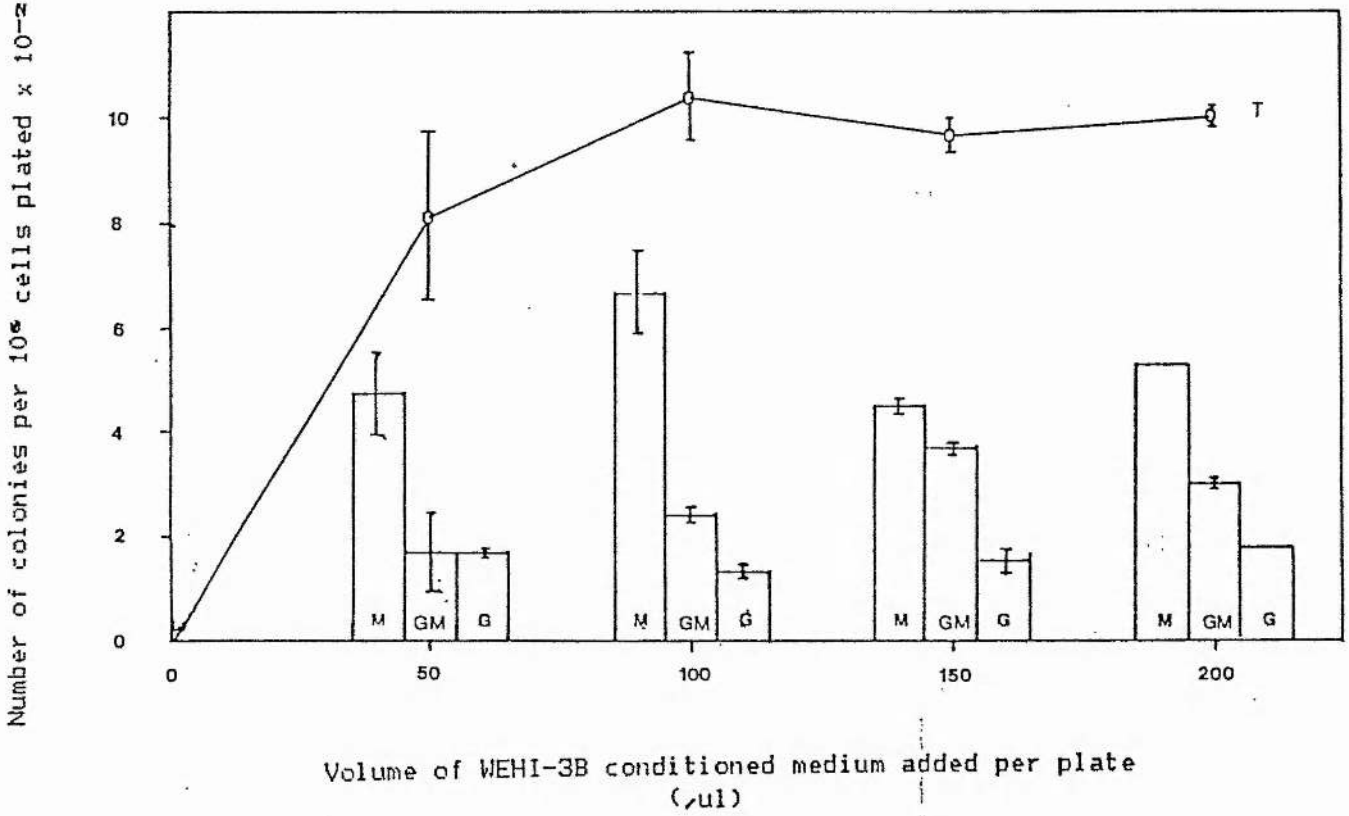
Table 3.1 suggests that there is a general decrease in the variation of number of colony type produced between groups with increasing CSA concentrations (F-test, $P < 0.1$). At lower concentrations of CSA, a high proportion of only macrophage colonies develop but at higher concentrations, a higher proportion of granulocyte-containing colonies were stimulated.

Further experiments using the *in vitro* GM-CFC agar culture assay employed a concentration of WEHI-3B conditioned medium of 9.1% (equivalent to the addition of 100 μl of CSA) per plate.

Table 3.1: Percentage of colony types stimulated in femoral bone marrow cells using the agar culture assay with increasing CSA concentrations. (M - macrophages, GM -granulocyte-macrophages and G - granulocyte).

Volume of CSA (/ul)	Percentage of colonies produced ± SE		
	Loose (M)	Mix (GM)	Tight (G)
50	58 ± 18	21 ± 10	21 ± 4
100	64 ± 10	23 ± 2	13 ± 1
150	46 ± 2	38 ± 1	16 ± 3
200	53 ± 0.3	30 ± 0.6	17 ± 0.01

Figure 3.1: The number of GM-CFC colonies produced in the agar culture assay with increasing amounts of WEHI-3B conditioned medium. Values are mean ± SE of four plates. G - Granulocytes (tight), M - Macrophages (loose), GM - Granulocyte-Macrophage and T - Total number of colonies.



3.1.1.2 Determination of the relationship between number of normal femoral bone marrow cells plated to the total number of colonies produced.

Experimental procedure

A femoral bone marrow cell suspension was prepared as described previously and normally plated using the agar culture system. Different cell doses were plated varying from 2.20×10^4 to 2.59×10^5 cells per plate and assayed for GM-CFC.

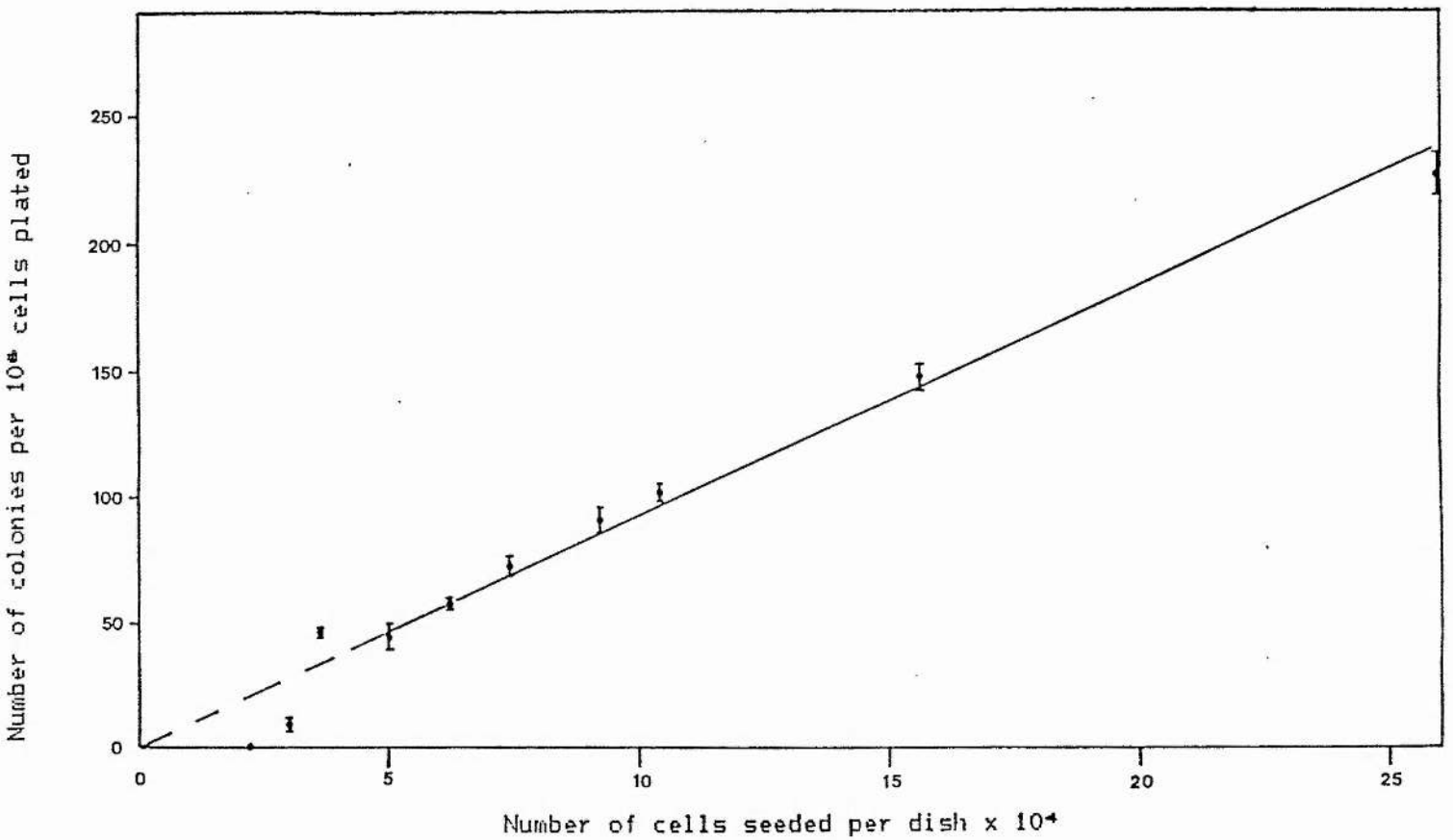
Results

The cell-dose response curve shown in Figure 3.2 illustrates that the normal murine femoral bone marrow cells exhibit a highly significant linear proliferative response (Regression analysis, $P < 0.001$) over the cell concentrations employed.

'Plating efficiency' is calculated to be 0.001% or 953 $\pm$ 14 (SEM) colonies produced per 10^6 cells seeded. The mean percentage ($\pm$ SEM) of different types of colonies formed were 78% ($\pm$ 4.4) for macrophagic (loose) colonies, 9.2% ($\pm$ 1.6) for granulocytic (tight) colonies and 12.2% ($\pm$ 3.0) for granulocyte-macrophage (mix) colonies. Difficulty was experienced in identifying the different types of colonies produced at the highest cell dose.

A cell dose between 5.0×10^4 to 2.0×10^5 were seeded per plate in subsequent agar culture assays as these would be expected to produce total GM-CFC colony counts of between 50 to 186 ($\pm$ 14) colonies respectively. In the radiation response determinations using the GM-CFC agar culture assay, additional cells were seeded to account for the reduction in GM-CFC survival.

Figure 3.2: The cell-dose response curve of the agar colony assay. Values are mean ± SE of five plates. The line is fitted by eye to the number of colonies data.



3.1.1.3 Effect of pre-incubation of normal femoral bone marrow cell suspension on the total number of colonies produced.

Experimental procedure

A femoral bone marrow cell suspension was prepared as described earlier and the cell concentration adjusted to 1.39 x 10^6 cells per ml suspension. This suspension was then immediately plated and this acted as the experimental control ie. where time t = 0. The cell suspension was then divided into two equal aliquots, one kept on ice (4^oC) and the other in a heated water-bath set at 37^oC. At specified intervals ie. t equals 1.0, 2.0, 3.5 and 6.0 hours, the cellularity of the cell suspension was determined and the suspension assayed for GM-CFC content using the agar culture assay.

Results

For both the incubation temperatures investigated, the GM-CFC content was slightly reduced when suspended in fully supplemented medium for 6 hours (T-test between experimental control is slightly significant to both tests when t = 6 hours ie. at 4^oC and 37^oC; $P < 0.1$; Table 3.2).

Figure 3.3A shows that GM-CFC number decreased less rapidly when kept on ice (4^oC) and was significantly higher at T = 6 hours (T-test at t = 6 hours; $P < 0.1$) ie. reduced 15% when kept on ice as compared to 21% on the heated water-bath (37^oC; Figure 3.3B). The decrease was not dependent on cell adherence to the walls of the container used. Figure 3.3C and 3.3D shows an increase in cell concentration at t = 2 hours, but at the same value of t, GM-CFC number were both reduced at both temperatures investigated (see Figure 3.3A and 3.3B).

In succeeding experiments, experimental controls were included in experimental designs using the agar culture assay which involved long incubation periods between preparation of cell suspension and plating. Cell suspensions were kept on ice, where possible; and, the time between preparation of cell suspensions and plating kept as short as possible.

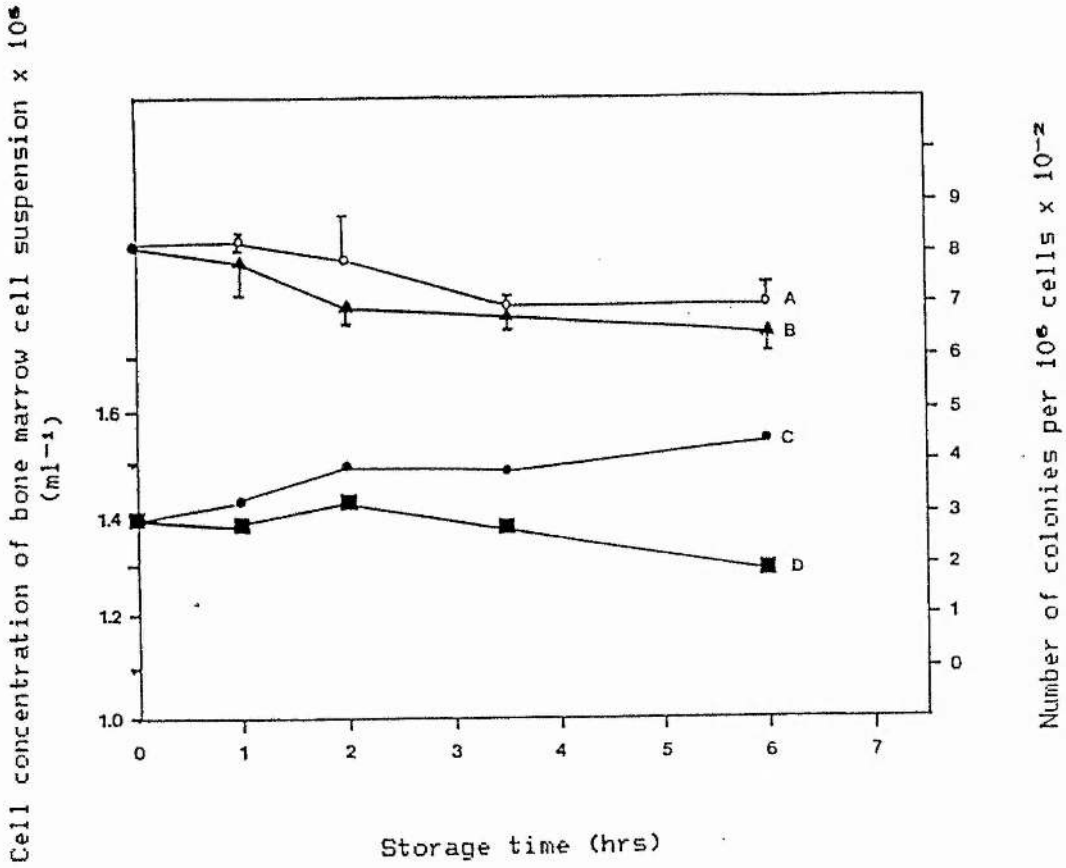
Table 3.2: Survival of femoral bone marrow GM-CFC on storage of cell suspension with time at different incubation temperatures ie. ice ($4^{o}C$) and in heated water-bath ($37^{o}C$). Values are expressed as a fraction of control at t = 0 hrs.

Time (hrs)	Survival fraction ± SE	
	$0^{o}C$	$37^{o}C$
1.0	1.00 ± 0.02	0.96 ± 0.08
2.0	0.96 ± 0.10	0.85 ± 0.04
3.5	0.85 ± 0.02	0.84 ± 0.02
6.0	0.85 ± 0.15	0.79 ± 0.04

Figure 3.3: The effect of storage of the bone marrow cell suspension to the cell concentration and to the production of GM-CFC colonies using the agar colony assay. Results are mean ± SE of four plates or readings.

A & B - number of GM-CFC colonies per 10^6 cells from cell suspension stored on ice (4°C) and in a heated water-bath (37°C) respectively.

C & D - cellularity of the suspension stored on ice (4°C) and in a heated water-bath (37°C) respectively.



3.1.2 Radioisotope uptake assay

3.1.2.1 Effect of different incubation conditions on the uptake of 5'-^{125}Iodo-2'-deoxyuridine (^{125}IUdR) by proliferating normal bone marrow cells.

Experimental procedure

A normal femoral bone marrow cell suspension was prepared in serum-free supplemented Fishers medium and the cell concentration adjusted to 1.48×10^7 cells per ml. Table 3.3A, 3.3B and 3.3C show the concentration of bone marrow cells, Foetal Calf Seum (FCS), and WEHI-3B conditioned medium of vials labelled 1 - 15. Unless stated otherwise, the final dose of cells plated was 1.03×10^5 cells and the final concentrations of FCS and WEHI-3B were both 10% (v/v). In vials 1 - 5, the WEHI-3B concentration was varied between 0 - 20%. The number of cells plated per plate in vials 6 - 10 was 0, 3.69×10^4, 1.47×10^5, 2.94×10^5 and 6.00×10^5 cells respectively. In vials 11 - 15, the FCS concentration was varied between 0 - 20%. ^{125}IUdR uptake was assayed as a measure of GM-CFC content.

Results

The standard error of mean of the Gamma-counter was $\pm$ 0.03% for a sample whose mean sample count for ^{125}IUdR was 1512 counts per minute.

In Figure 3.4A, it was evident that the incorporation ^{125}IUdR to the cells in the absence of WEHI-3B conditioned medium was just significantly different from background (T-test, $P < 0.01$). 125IUdR incorporation increases rapidly with increasing WEHI-3B concentrations as noted by the increase in the activity measured and gradually plateaus after 10 % CSA concentration (T-

Table 3.3: Table showing the different volumes of cell suspension, Foetal Calf Serum (FCS), WEHI-3B conditioned medium and additional Fischers medium used in vials 1 - 15 (Total volume = 2 mls.) Vials 1 - 6, B = Vials 6 - 10, C = Vials 11 - 15.

A

Vial No:	1	2	3	4	5
WEHI-3B (/ul)	0	50	100	200	400
(%)	0	2.5	5.0	10.0	20.0
Cells (/ul)	70	70	70	70	70
FCS (/ul)	200	200	200	200	200
Fischers med. (/ul)	1730	1680	1630	1530	1330

B

Vial No:	6	7	8	9	10
Cells (/ul)	0	50	100	200	400
(ml^{-1}/ x 10^5)	0	3.7	7.4	1.5 x 10	5.0 x 10
FCS (/ul)	70	70	70	70	70
WEHI-3B (/ul)	200	200	200	200	200
Fischers med. (/ul)	1600	1550	1500	1400	1200

C

Vial No:	11	12	13	14	15
FCS (/ul)	0	50	100	200	400
(%)	0	2.5	5.0	10.0	20.0
Cells (/ul)	70	70	70	70	70
WEHI-3B(/ul)	200	200	200	200	200
Fischers med. (/ul)	1730	1680	1630	1530	1330

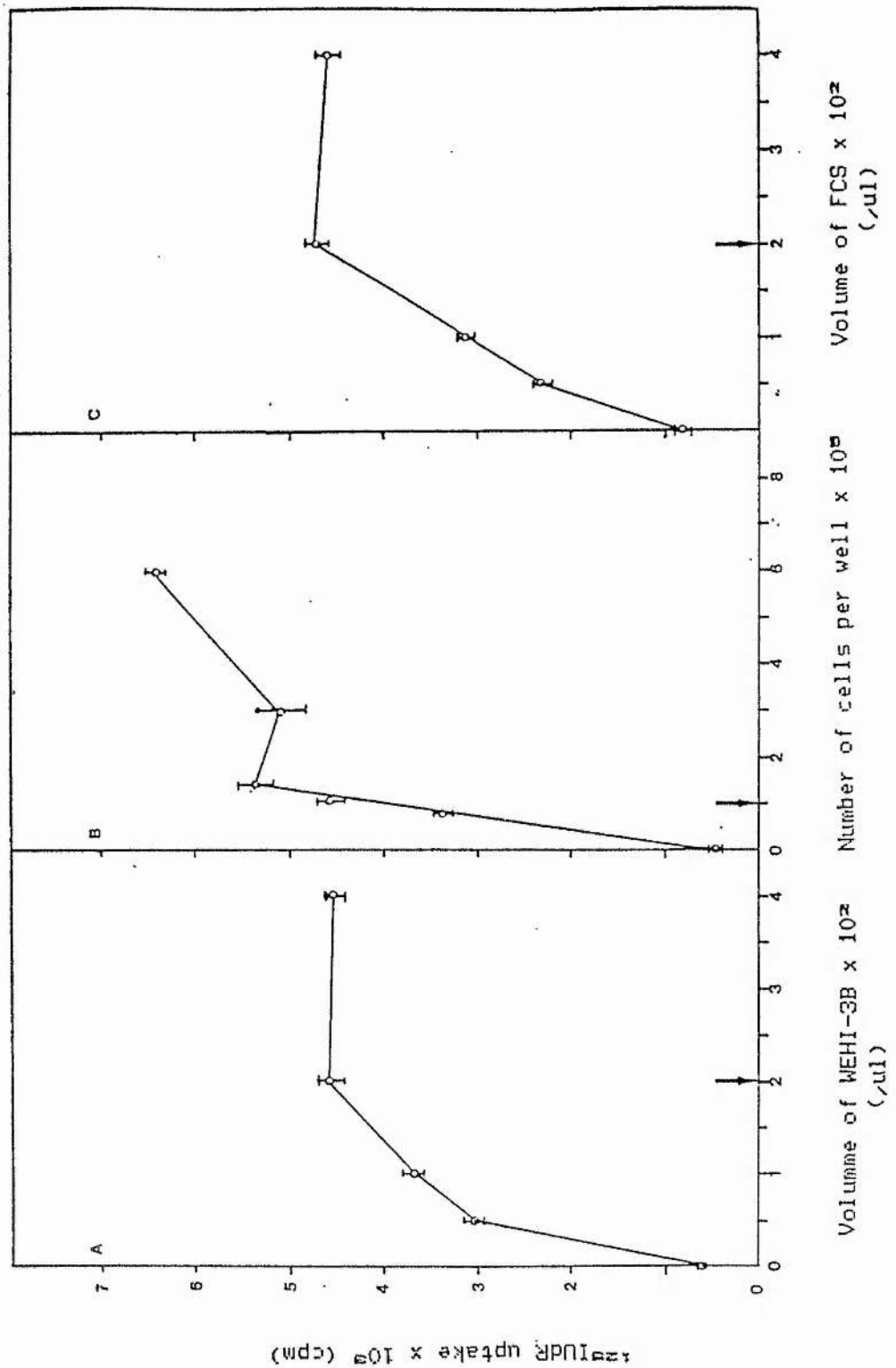
test of the two highest concentrations show no significant difference, $P < 0.05$).

Response of incorporation was significantly linear with cell dose between 0 to 1.03×10^5 cells per plate (Regression analysis, $P < 0.001$). With increasing number of cells plated, the incorporation of ^{125}IUdR gradually plateaus (Figure 3.4B).

The incorporation of ^{125}IUdR by proliferating femoral bone marrow cells in the presence of WEHI-3B was maximum when the FCS concentration was 10% and incorporation of ^{125}IUdR decreased when FCS concentration was further increased (Figure 3.4C, T-test of the two highest concentrations show slightly significant differences, $P < 0.05$).

Subsequently, the measurement of proliferative activity using the radioisotope uptake assay employed FCS and WEHI-3B concentrations of 10% each. Approximately 1×10^5 cells were plated and in the radiation response experiments, an additional plate was plated having a cell dose of 2×10^5 cells.

Figure 3.4: Radioistope uptake in proliferating femoral bone cells against A) increasing concentrations of WEHI-3B conditioned medium, B) increasing number of cells plated per well and C) increasing concentrations of Foetal Calf Serum (FCS).
Values are mean ± SE of 9 plates. ↓ indicate optimal concentrations.



3.2 RADIATION RESPONSES OF NORMAL BONE MARROW GRANULOCYTE-MACROPHAGE PROGENITOR CELLS

3.2.1 Radiation responses of GM-CFC in femoral bone marrow of mice irradiated in vivo (whole-body irradiation).

Experimental procedure

Groups of three mice were irradiated with 0.5, 1.0, 1.5, 2.0 and 3.0 Gy dose of X-rays. At each dose the marrow from a single femur from each mouse were pooled and assayed for GM-CFC content by the agar culture assay. Marrow from the remaining femurs were also pooled and assayed for its proliferative capacity by the radioisotope uptake assay using ^{3}H-Thymidine (^{3}HTdR). Pooled bone marrow cells from unirradiated were used as controls (ie. 0 Gy).

Results

In neither the agar culture assay nor the radioisotope uptake assay were there any evidence of colonies present or the incorporation of ^{3}HTdR in cells assayed without WEHI-3B. This was observed for all the irradiation doses investigated.

In addition, the number of colony-forming units in the agar culture assay were also expressed as per mean femur and calculated as a fraction of the control (unirradiated) values.

From Figures 3.5A and 3.5B, the dose survival curves using both the agar culture assay for GM-CFC and the radioisotope uptake assay showed significantly negative log-linear relationship (Regression analysis, F-test, both $P < 0.001$) and no evidence of a shoulder (estimated value of n approximates unity; see Table 3.4).

There was also a significant difference in the coefficient of regressions (Comparison of regression coefficients; $P < 0.01$) of

the relationship between the survival calculated per femur and to per 10^6 cells in the agar culture assay. However there was a strong correlation between the two values (Correlation analysis; $P < 0.001$). There was also strong correlation between the radioisotope uptake data and the agar culture data (Correlation analysis, $P < 0.001$). A comparison of the regression coefficients and of both the assays suggest that the viable cells measured by the radioisotope assay appears to be significantly less sensitive to radiation than those measured by the agar clonal assay ($P > 0.05$) and this is reflected by the D_o values.

The values of n, mean D_o and regression coefficients are shown in Table 3.4.

Figure 3.5A: Radiation survival curve of GM-CFC from femoral bone marrow irradiated in vivo using the agar culture assay. Line is fitted by eye to the survival fraction data only. Results are mean ± SEM of three experiments.

● - Survival fraction expressed as per femur

△- Survival fraction epressed as per 10^4 cells plated

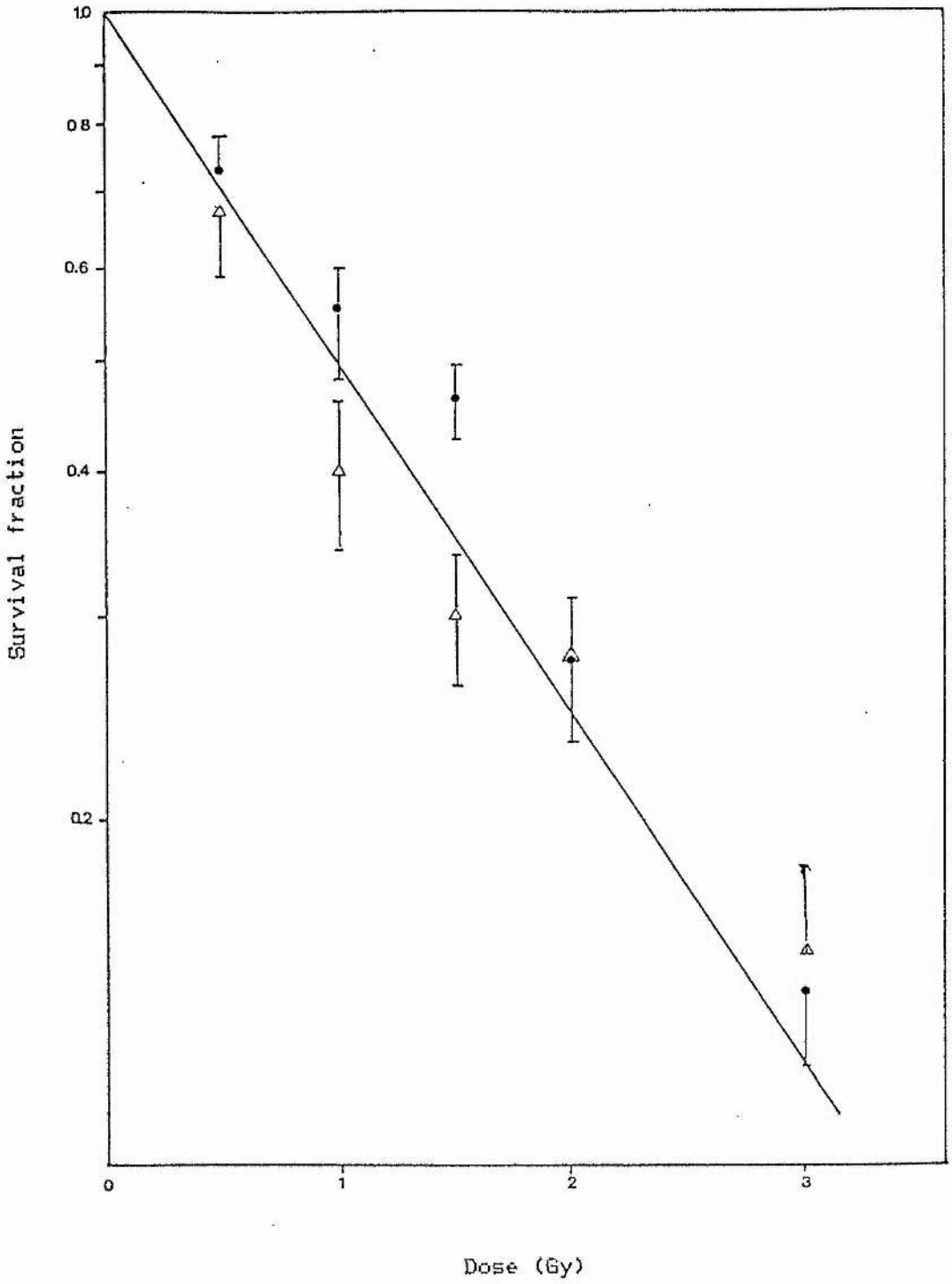


Figure 3.5B: Comparison of the survival curves of GM-CFC from femoral bone marrow irradiated in vivo using the agar culture assay (△) and the ^{3}HTdR uptake assay (●). Values are mean ± SEM of three experiments. Lines are fitted by eye to the surviving fraction data only.

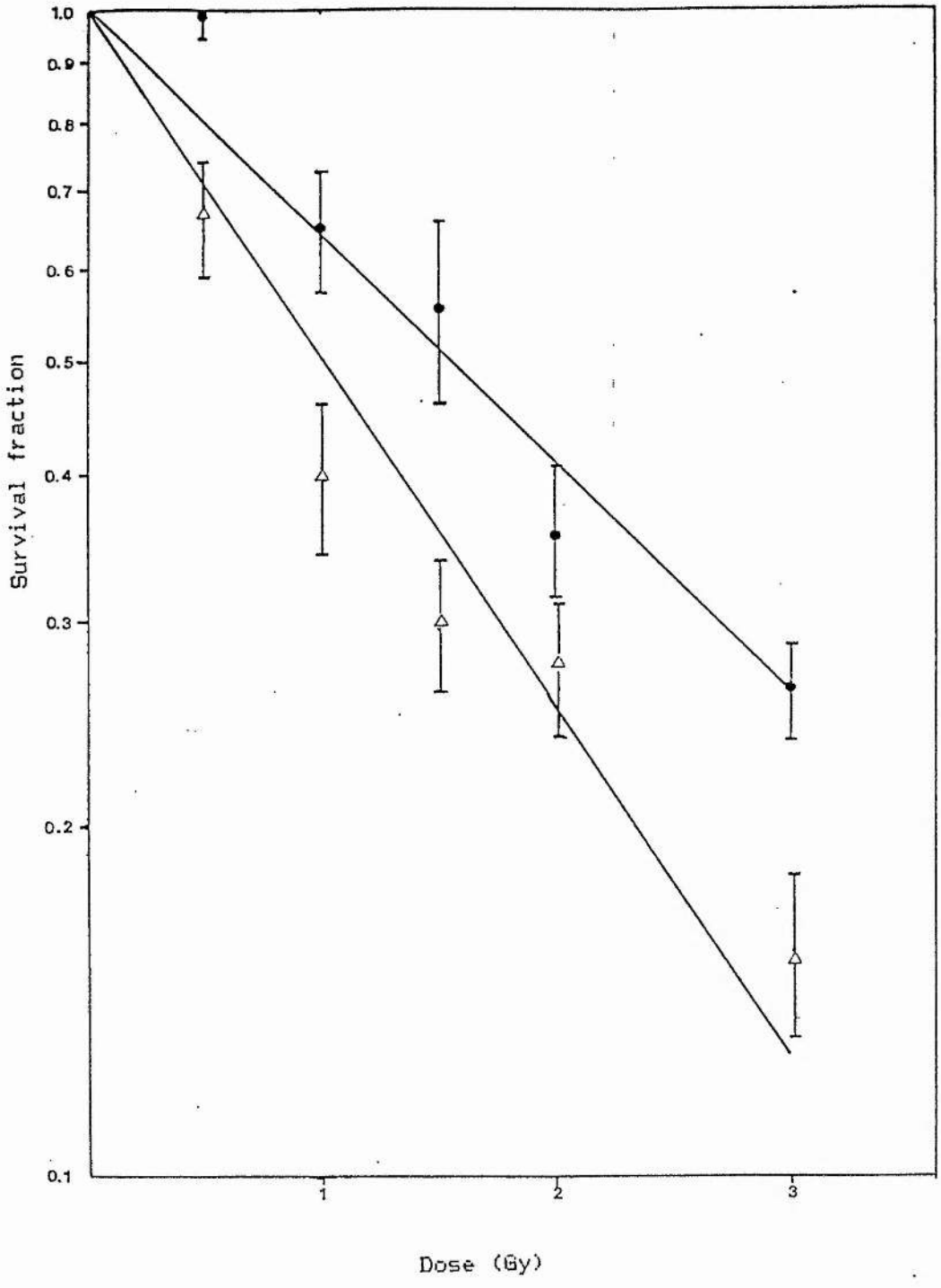


Table 3.4: Survival characteristics of murine femoral bone marrow GM-CFC after acute doses of X-rays. Values are means ± SE and obtained using the SUPERSTATS™ statistical program on the APPLE II+ personal computer (64K).

GM-CFC assay	Irradiation condition	Survival fraction	D_o (Gy)	n
Agar culture	in vivo	per femur	1.03 ± 0.07	1.15 ± 0.06
		per 10^6 cells	1.24 ± 0.04	1.04 ± 0.04
	in vitro	per 10^6 cells	1.00 ± 0.09	1.08 ± 0.09
Radio-isotope uptake	in vivo	per 10^6 cells	1.74 ± 0.09	1.09 ± 0.05
	in vitro	per 10^6 cells	2.10 ± 0.10	1.12 ± 0.06

3.2.2 Radiation responses of GM-CFC from the femoral bone marrow of mice irradiated *in vitro*.

Experimental procedure

Pooled bone marrow cells were irradiated with 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 and 8.0 Gy dose X-rays. The cell suspension were assayed for GM-CFC content using the agar culture assay and the radioisotope uptake assay employing 5'-^{125}Iodo-2'-deoxyuridine (^{125}IUdR).

Results

From Figure 3.6, we conclude that using the agar culture asay, the survival dose response curves shows a highly significant log-linear relationship (Regression analysis and F-test; both $P < 0.001$) and no evidence of a shoulder (Regression analysis, value of n approximates unity; Table 3.4).

CSA controls show no evidence of colonies or clusters present in the agar culture assay nor were there any incorporation of ^{125}IUdR at any of the dose levels investigated.

As in the *in vivo* irradiation studies, the data shows that the viable cells measured by the radioisotope uptake assay are less sensitive to irradiation than to those measured by the agar clonal assay (see D_o values in Table 3.4; comparison of the regression coefficients showed significant difference, $P < 0.1$)

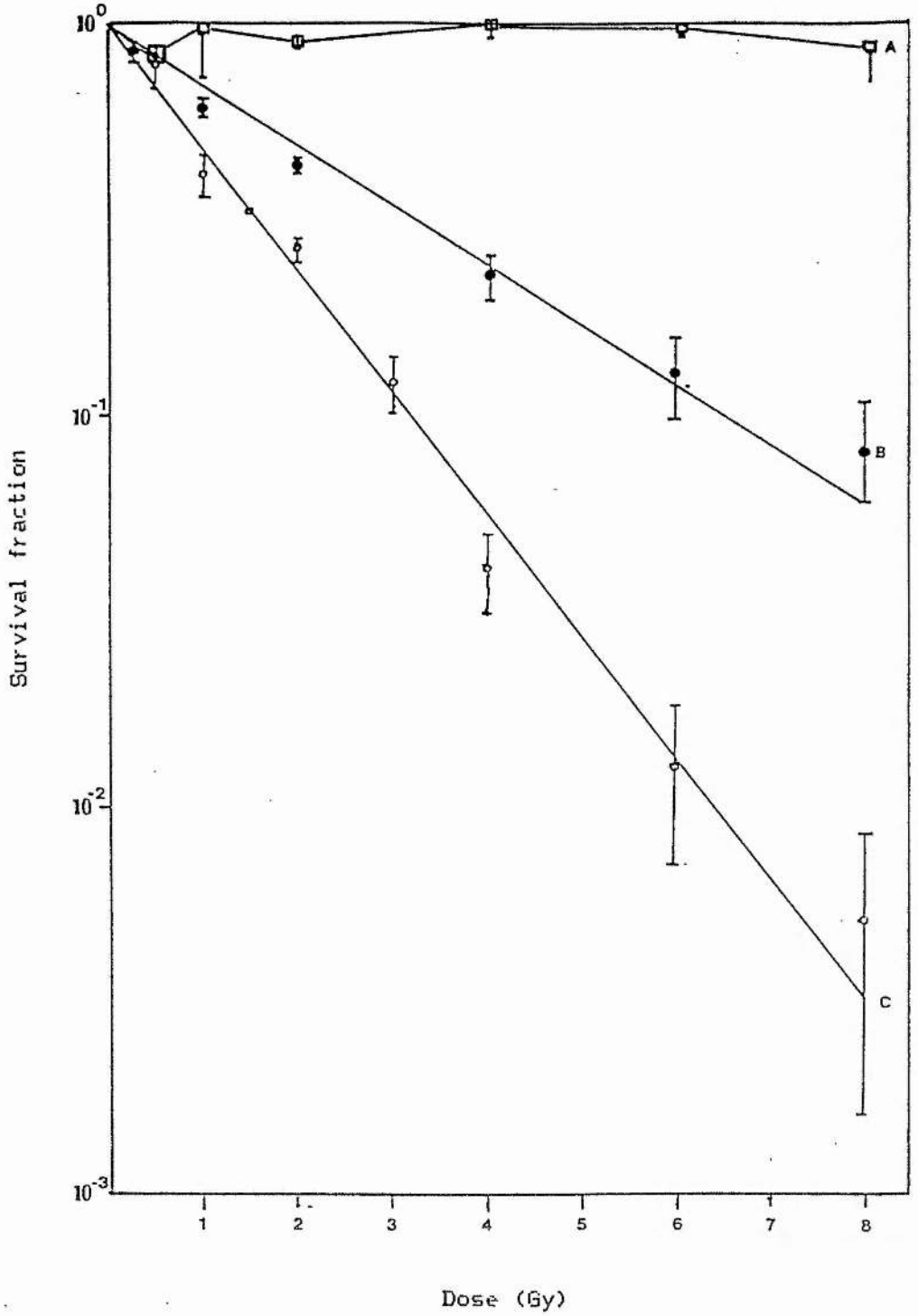
The correlation coefficient between the two assays were highly significant (Correlation analysis, $P < 0.001$).

Figure 3.6: Radiation survival curves of GM-CFC from femoral bone marrow cells irradiated in vitro using the agar culture assay and the ^{125}IUdR uptake assay. Results are mean ± SEM of a minimum of three experiments.

A - ^{125}IUdR uptake in the absence of WEHI-3B (CSA controls)

B - ^{125}IUdR uptake assay.

C - Agar culutre assay.



3.2.3 Effect of concentration of colony stimulating activity (CSA) on the number of GM-CFC of irradiated femoral bone marrow.

Experimental procedure

Pooled femoral bone marrow cells were irradiated with 0, 1, 2, and 3 Gy dose of X-rays. GM-CFC was assayed using the agar culture system with either 25, 50, 100 or 200 /ul of WEHI-3B conditioned medium added per plate. The final volume was kept constant at 1.2 mls with the addition of appropriate volumes of buffered medium. A colony is defined as containing 40 - 50 cells and smaller clones termed clusters as having lesser number of cells.

Results

Figure 3.7 shows that for the doses of X-rays investigated, the total number of colonies produced per 10^6 cells seeded plateaus after 100 or more /ul of CSA was added per plate (T-test between 100 /ul amd 200 /ul CSA added at each X-ray does not any show significant difference, $P > 0.1$)

The curve shown on Figure 3.8 was obtained by normalising the data to the maximum number of colonies produced at each X-ray dose irradiated. It shows that there is no significant difference when either 100 /ul or 200 /ul of CSA were used (F-test, $P > 0.1$). The difference between the cluster-colony ratio obtained with the different CSA concentrations used was significant (F-test, $P < 0.1$; Table 3.5). Examination of the decrease in cluster number does not show corresponding numerical increase in colony formation. But in general, the ratio plateaus at all doses of X-rays administered with the higher concentration of CSA (T-test at individual X-ray doses between the two higher CSA concentrations show no significant difference, $P > 0.1$).

Figure 3.7: Effects of CSA on the number of GM-CFC from irradiated femoral bone marrow cells. Results are mean ± SE of four plates in a single experiment.

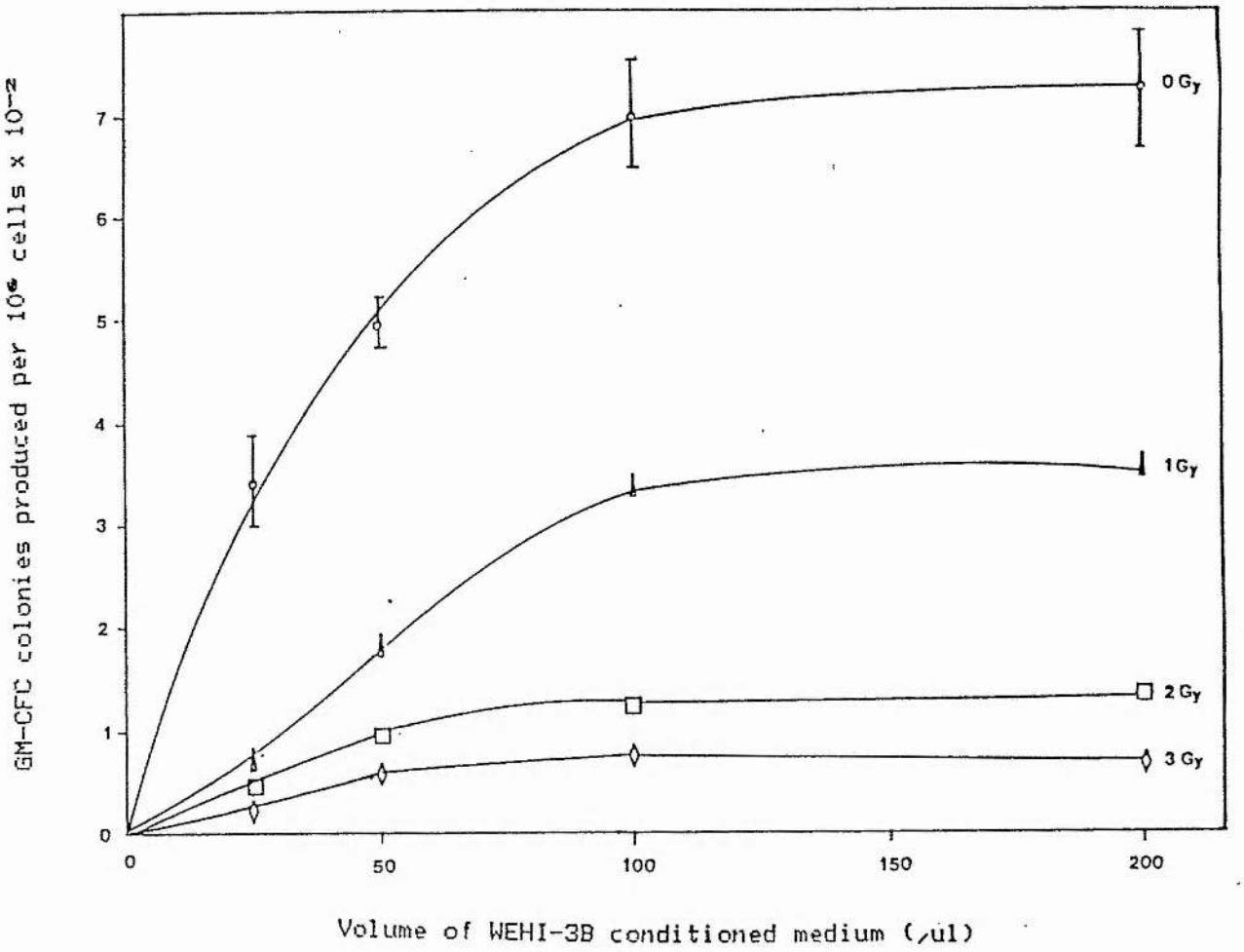


Figure 3.8: Effects of CSA on the percentage of the maximum number of GM-CFC from irradiated femoral bone marrow cells.

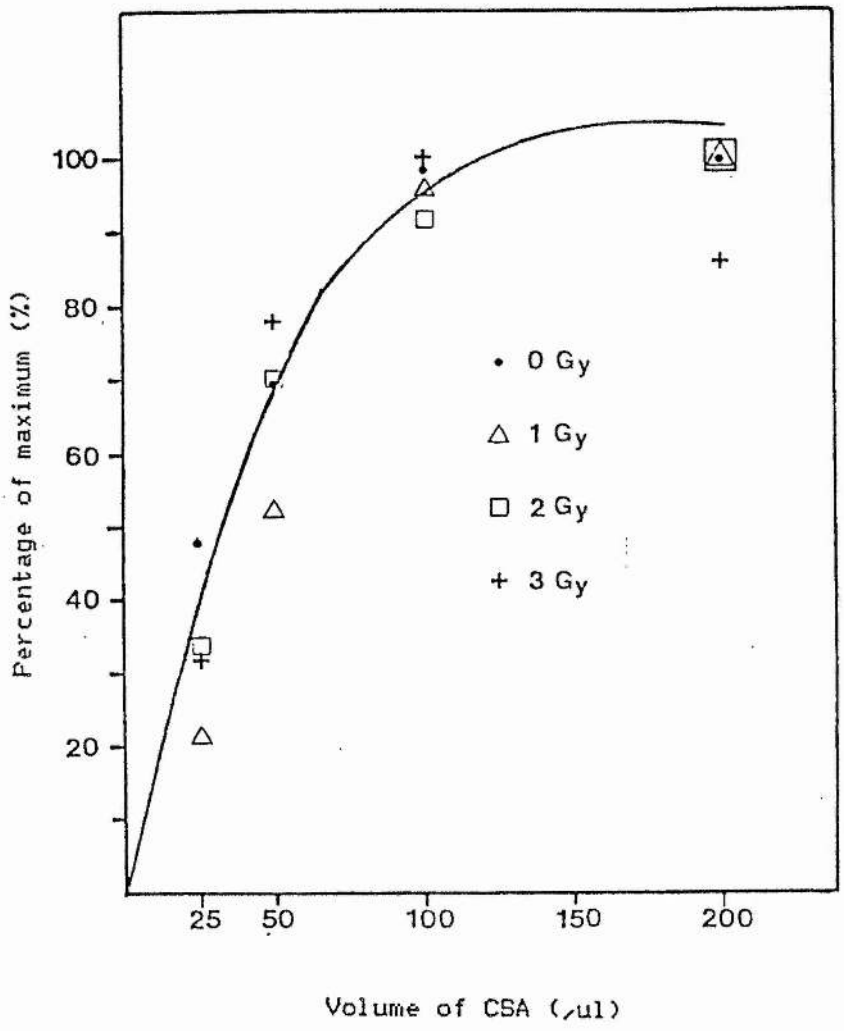


Table 3.5: Colony and cluster formation in normal and X-irradiated femoral bone marrow cell cultures with increasing amount of added CSA. Survival fractions were expressed as a fraction of non-irradiated controls. Values represent the mean ± SE of four plates.

Dose (Gy)	Volume of CSA (/ul)	GM-CFC per 10^6 cells	Survival fraction	Clusters per 10^6 cells	Cluster-Colony ratio
	0	-	-	-	-
	25	342 ± 44	control	506 ± 60	1.48 ± 0.25
0	50	497 ± 24	control	497 ± 36	1.00 ± 0.09
	100	700 ± 55	control	475 ± 55	0.68 ± 0.09
	200	722 ± 56	control	501 ± 58	0.69 ± 0.10
	0	-	-	-	-
	25	73 ± 8	0.21 ± 0.04	409 ± 37	5.60 ± 0.76
1	50	181 ± 14	0.36 ± 0.03	243 ± 21	1.34 ± 0.15
	100	336 ± 22	0.48 ± 0.05	255 ± 45	0.76 ± 0.14
	200	351 ± 15	0.48 ± 0.04	317 ± 27	0.90 ± 0.09
	0	-	-	-	-
	25	46 ± 9	0.13 ± 0.03	203 ± 26	4.42 ± 1.04
2	50	94 ± 2	0.19 ± 0.01	125 ± 21	1.32 ± 0.22
	100	124 ± 4	0.18 ± 0.02	148 ± 19	1.19 ± 0.15
	200	134 ± 5	0.18 ± 0.02	161 ± 13	1.20 ± 0.11
	0	-	-	-	-
	25	24 ± 2	0.07 ± 0.01	na	na
3	50	59 ± 5	0.12 ± 0.01	na	na
	100	76 ± 7	0.11 ± 0.01	na	na
	200	65 ± 6	0.09 ± 0.01	na	na

na - Not available

3.2.4 Response of femoral bone marrow GM-CFC to a split-dose of X-rays.

Experimental procedure

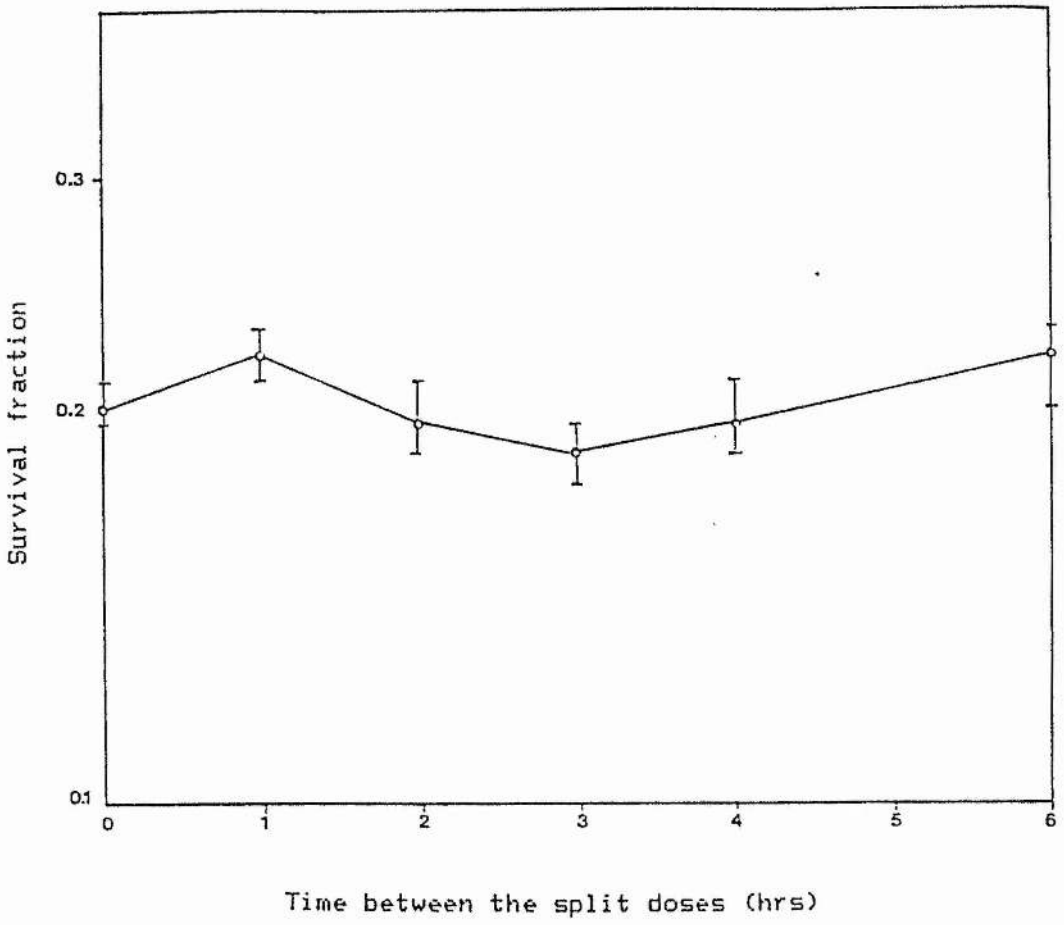
Pooled bone marrow ells were irradiated with a split-dose of 2 x 1.0 Gy X-rays. Cell suspensions were incubated in a heated water-bath ($37^{o}C$) and the GM-CFC assayed using the agar culture technique.

Results

Figure 3.9 showed the relationship of GM-CFC survival expressed as a fraction of single dose (2.0 Gy) control to the duration between the two doses. Analysis of variance within and between groups show no significant differences (F-test, $P > 0.1$). Murine femoral bone marrow GM-CFC show no recovery or increase in radiation resistance in the duration between the two doses of X-rays at the temperature investigated.

In preliminary experiments not reported here, similar results were obtained when the same experiment was performed with the cell suspension incubated in ice ($4^{o}C$) between the two doses of X-rays.

Figure 3.9: Recovery of GM-CFC from femoral bone marrow irradiated with a split dose regimen (2 x 1 Gy). Values are mean ± SE of three plates.



3.3 TRYPSINISATION EXPERIMENTS

3.3.1 The effects of procedures involved in the trypsinisation process on femoral bone marrow GM-CFC survival.

Experimental procedure

A pooled femoral bone marrow cell suspension was prepared in serum-free suplemented Fischers medium. Two ml. aliquots were run through the different stages of the sequence of the trypsinisation process. Following each treatment of normal bone marrow cells listed below, the cell suspension was incubated in a heated water-bath ($37^{o}C$) for 10 mins.

A. Preparation of cell suspension (no treatment control).

B. Cells spun at 1000 rpm for 15 mins. and resuspended in the same medium.

C. Same as in B but resuspended in two mls. of fresh medium.

D. Same as in B but resuspened in two mls. of PBS buffer containing no EDTA.

E. Same as in D but in PBS-EDTA buffer.

F. Same as in D but in PBS-EDTA buffer containing 0.05% trypsin (v/v).

In treatments D, E and F, 200 /ul of horse serum was added to the cells at the end of incubation. The cells were further spun at 1000 rpm for 15 mins. and resuspended with appropriate volumes of fully supplemented Dulbecco's medium. GM-CFC was assayed using the agar culture assay.

Results

There were no significant differences in GM-CFC survival between cells treated with treatments A to E ((F-test, $P > 0.05$;

Table 3.6).

However, there was a highly significant difference between the GM-CFC content of the no treatment control and the trypsinised sample (T-test, $P < 0.001$). Trypsinisation decreased the number of GM-CFC to 54% $\pm$ 3.

The data suggests that the perturbations involved in the trypsinisation procedure does not affect the survival of GM-CFC. GM-CFC killing is due only to the presence of trypsin.

Table 3.6: Effect of different stages of the trypsinisation procedure on femoral bone marrow survival. Data are mean ± SE of four plates.

Treatment label	Treatment investigated	Total No: of GM-CFC per 10^6 cells seeded	Percentage control
A	No treatment control	1015 ± 33	100 ± 3
B	Spinning 1000 rpm for 15 mins	1002 ± 39	99 ± 5
C	Change of medium after spinning	989 ± 24	97 ± 4
D	Change of buffer to PBS	862 ± 55	85 ± 6
E	EDTA in PBS	1051 ± 56	103 ± 7
F	0.05% trypsin-PBS-EDTA buffer	544 ± 28	54 ± 3

3.3.2 The effect on femoral bone marrow GM-CFC survival following different incubation conditions during trypsinisation.

Experimental procedure

Pooled bone marrow cells were incubated with trypsin for 5 and 10 mins. in a heated water-bath (37^{o}C).

Pelleted marrow cells were trypsinised with 0.05% trypsin either with or without an additional pre-washing procedure. Pre-washing involved the addition of 2 mls. of PBS-EDTA-0.05% trypsin buffer in which the cells were resuspended by gentle agitation. The trypsin buffer was then immediately removed without any incubation followed by spinning at 1000 rpm for 15 mins. The pre-washed cells were then trypsinised normally.

Bone marrow cells were also trypsinised (without pre-washing) with 0.025 and 0.05% trypsin (10 mins., 37^{o}C) and to the resulting pellet of cells was added 3 /ul of restriction endonuclease storage buffer and incubated in a heated water-bath (37^{o}C) for 30 mins. Medium was substituted for storage buffer to act as buffer controls. The treated cells were resuspended in fully supplemented Dulbecco's medium and assayed for GM-CFC using the agar culture assay.

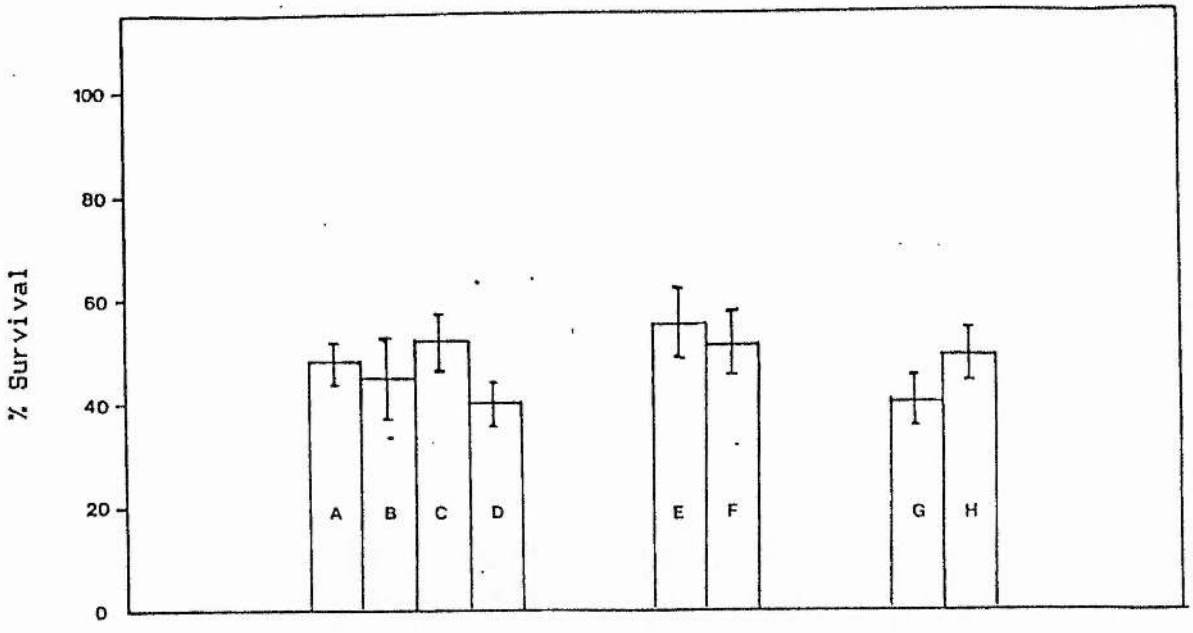
Results

There was no significant difference between the percentage of GM-CFC survival with cells trypsinised with or without a pre-washing procedure regardless of the period incubated (F-test, $P > 0.05$; see figure 3.10)

Subsequently, in experiments requiring trypsinisation, cells were incubated for 10 mins. in the heated water-bath (37^{o}C) and did not include a pre-washing step.

Data obtained from the preliminary experiments of the effect of 3 μl of storage buffer on GM-CFC survival of cells pretreated with either 0.025 or 0.05% trypsin showed no significant difference with their buffer controls (T-test, $P > 0.1$). There was no significant difference of the data when it was obtained with either 0.05 or 0.025% trypsin (F-test, $P > 0.1$; Figure 3.10 E, F, G and H).

Figure 3.10: Effects of different trypsin concentrations and different incubation conditions on femoral bone marrow GM-CFC. Data are mean ± SE of four plates.



A - 0.05 % trypsin, 37°C, 5 mins.,pre-washed.

B - 0.05 % trypsin, 37°C, 5 mins., no pre-washing.

C - 0.05 % trypsin, 37°C, 10 mins.,pre-washed.

D - 0.05 % trypsin, 37°C, 10 mins., no pre-washing.

E - 0.025 % trypsin, 37°C, 10 mins.; medium (buffer control), 30 mins., 37°C, 3 ,ul.

F - 0.025 % trypsin, 37°C, 10 mins.; Storage buffer, 30 mins., 37°C, 3 ,ul.

G - 0.05 % trypsin, 37°C, 10 mins.; medium (buffer control), 30 mins., 37°C, 3 ,ul.

H - 0.05 % trypsin, 37°C, 10 mins.; Storage buffer, 30 mins., 37°C, 3 ,ul.

3.3.3 The effect of increasing concentrations of trypsin on femoral bone marrow GM-CFC survival.

Experimental procedure

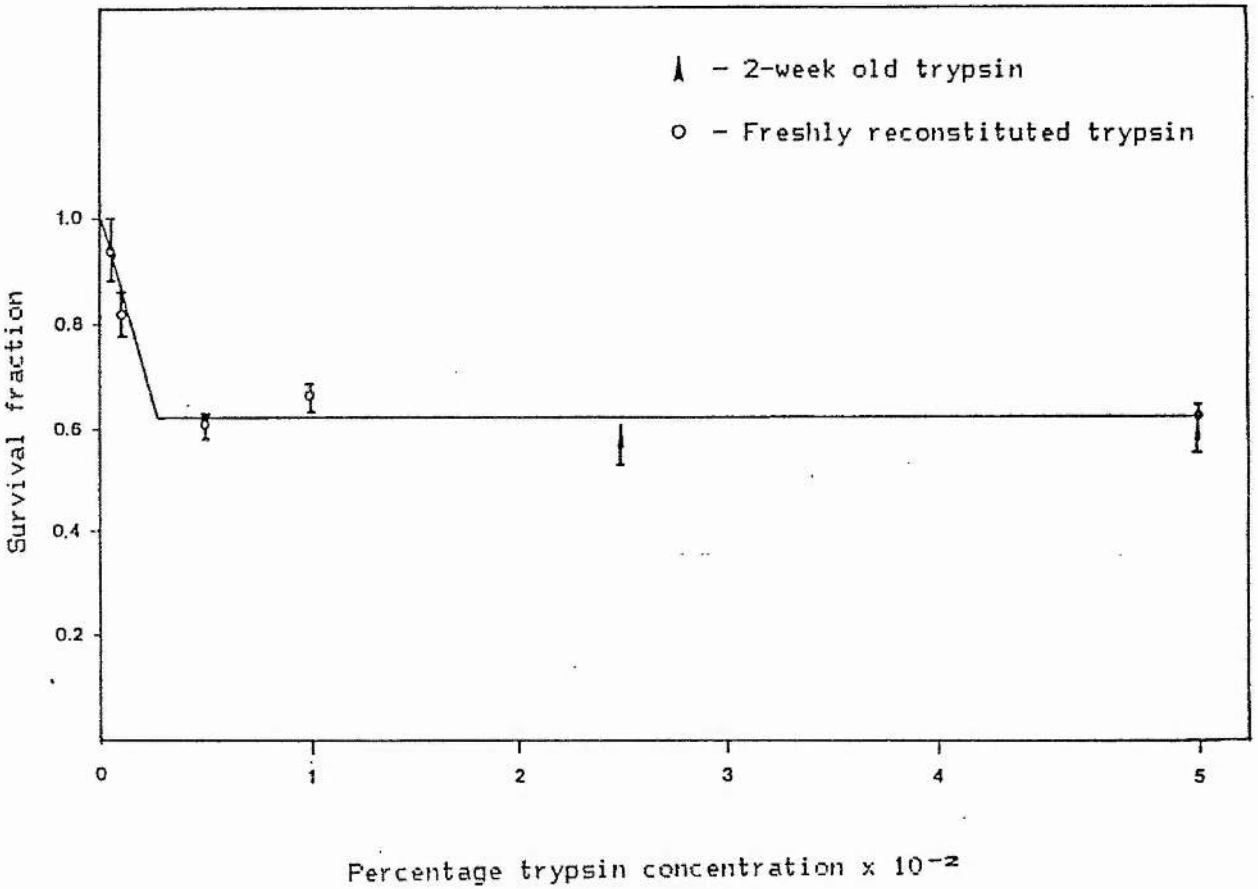
Femoral bone marrow cells were incubated with 1×10^{-4}, 5×10^{-3}, 1×10^{-2} and 5×10^{-2}% trypsin for 10 mins in a heated water-bath ($37^{o}C$). For the highest concentration of trypsin used, two-week old and freshly-reconstituted solutions were compared. GM-CFC was assayed using the agar culture assay.

Results

Figure 3.11 shows the relationship of GM-CFC survival following treatment with increasing concentrations of trypsin. The relationship between GM-CFC killing and trypsin concentration shows a plateau.

A significant rectilinear component (Regression analysis, $P < 0.001$) was evident between trypsin concentrations 0 to 0.005% and further increase in concentration show deviation from linearity (Regression analysis, F-test, $P < 0.01$). Examination of the scatter diagram shows that after 0.005% trypsin, the survival of GM-CFC was not significantly different with further increase in concentration of trypsin (F-test, $P > 0.05$). There was a highly significant difference in GM-CFC survival between marrow cells trypsinised with 0.001 and 0.05% trypsin (T-test, $P < 0.01$). Additional data on Figure 3.11 showed that there was no significant difference between surviving proportion of GM-CFC of marrow cells trypsinised with either freshly reconstituted or two-week old 0.05% trypsin (T-test, $P > 0.1$).

Figure 3.11: Effects of different concentrations of trypsin on the survival fraction of femoral bone marrow cell GM-CFC. Data are mean ± SE of five plates.



3.3.4 Recovery of femoral bone marrow GM-CFC following trypsinisation.

Experimental procedure

Pooled bone marrow cells were trypsinised with 0.05% trypsin. Following treatment, cells were resuspended in serum-free supplemented Fischers medium and incubated in a heated water-bath (37^{o}C). At specific intervals of 0, 1, 2, 3, 4, and 6 hours after trypsinisation, the suspension was assayed for GM-CFC content using the agar culture technique.

Results

There was no significant change of survival fraction between groups plated at specific intervals after trypsinisation (F-test, $P > 0.05$). However, there was a significant difference between the no-treatment controls and between the trypsinised cell with time (F-test, $P < 0.01$ for both; Table 3.7).

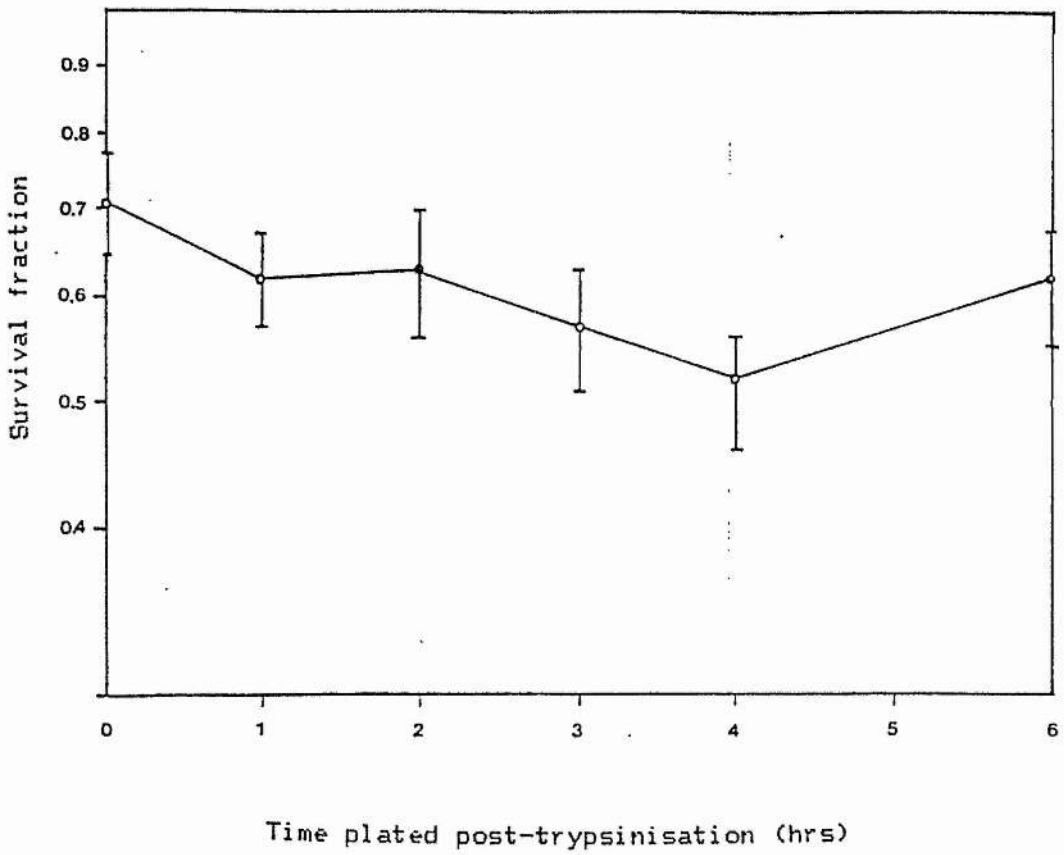
Figure 3.12 showed no significant log-linear relationship between the survival fractions observed at different times post-trypsinisation (Regression analysis, $P > 0.1$).

Similar experiments with 0.01% trypsin not reported here showed similar results.

Table 3.7: Recovery of GM-CFC of femoral bone marrow following trypsinisation with 0.05% trypsin (37°C, 10 mins.).

Time after trypsinisation (hrs)	Total No: of GM-CFC ± SE per 10^6 cells seeded	
	Control	Test
0	846 ± 21	600 ± 47
1	846 ± 21	524 ± 42
2	711 ± 51	449 ± 34
3	787 ± 75	449 ± 28
4	718 ± 53	376 ± 16
6	473 ± 10	294 ± 22

Figure 3.12: Recovery of femoral bone marrow GM-CFC following trypsinisation (0.05 %). Results are means ± SE of four plates in a single experiment.



3.3.5 Determination of the proportion of trypsin-sensitive femoral bone marrow GM-CFC in DNA synthesis (ie. in S-phase).

Experimental procedure

Femoral bone marrow cells in DNA-synthesis (S-phase) were measured using a suicide technique. Trypsinised and untreated cells were incubated with an S-phase specific drug ARA-C for 60 mins. in a heated water-bath (37^{o}C). The drug was then removed by washing the cells several times with fresh medium. The suicide technique was also used to prepare S-phase deficient bone marrow cells which were immediately trypsinised using the same trypsin concentration. The trypsin concentration used was 0.05% (10 mins., 37oC). GM-CFC was assayed using the agar culture assay.

Results

Table 3.9 shows the mean GM-CFC survival fractions of both the treated and untreated marrow cells. The proportion of GM-CFC sensitive to trypsin between that which were treated with ARA-C (S-phase cell deficient) and to those which were not treated was not significantly different (T-test, $P > 0.05$).

The percentage of normal murine femoral bone marrow GM-CFC actively synthesising DNA was 31% and this was not significantly different to the percentage of GM-CFC in DNA-synthesis in pre-trypsinised cells (T-test, $P > 0.1$).

However, the total number of GM-CFC produced by either the treatment of ARA-C followed by trypsin or vice versa was significantly different (T-test, $P < 0.01$), the former being greater. It was also interesting to note that the expected number of GM-CFC produced following both the treatments was estimated to be 0.31 ± 0.09 and was not significantly different to the observed value (T-test, $P > 0.1$ for both).

Table 3.8: Effect of 0.05% trypsin (37°C, 10 mins.) or ARA-C (37°C, 30 mins.) or the combination of both on the survival of femoral bone marrow GM-CFC.

Treatment	Total No: of GM-CFC ± SE per 10^6 cells		Survival fraction
	Control	Test	
Trypsin only	977 ± 21	436 ± 17	0.45 ± 0.02
ARA-C	925 ± 17	642 ± 24	0.69 ± 0.03
Trypsin - ARA-C	953 ± 12	333 ± 8	0.34 ± 0.01 [1](0.76 ± 0.04)
ARA-C - Trypsin	958 ± 89	266 ± 9	0.27 ± 0.03 [2](0.41 ± 0.02)

1 Fraction of Trypsin only.
2 Fraction of ARA-C only.

3.3.6 The response of femoral bone marrow GM-CFC to a single dose of X-rays administered at various intervals following trypsinisation.

Experimental procedure

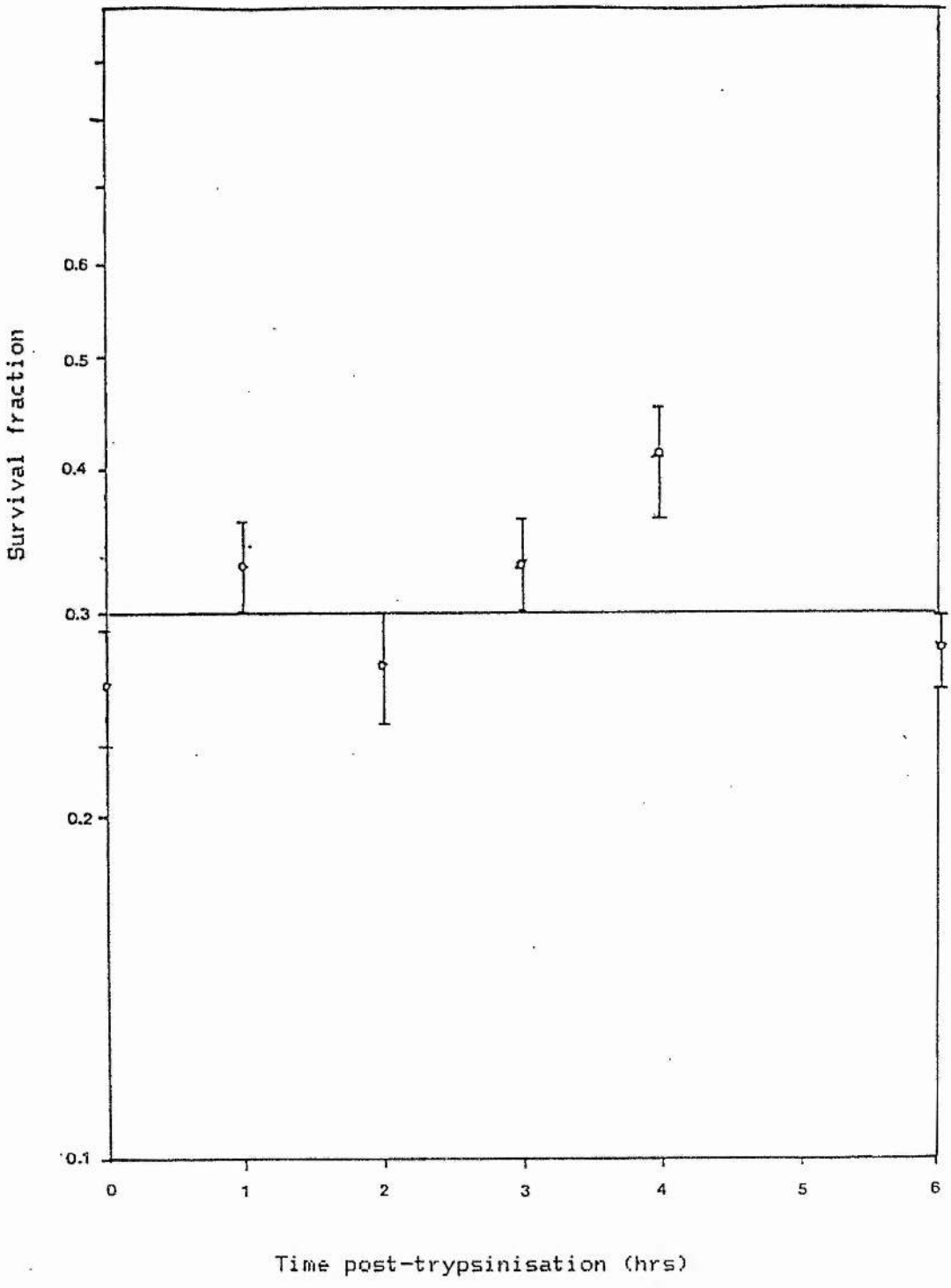
Femoral bone marrow cells were treated with 0.05% trypsin (10 mins, 37^{o}C) and resuspended in Fischers medium. The pre-trypsinised cells were then irradiated with a single dose of 2 Gy of X-rays at various time intervals ie. 0, 1.0, 2.0, 4.0 and 6.0 hours following pre-trypsinisation. The pre-trypsinised cell were incubated in a heated water-bath (37^{o}) prior to irradiation after which they were immediately plated. GM-CFC was assayed along with corresponding trypsin controls and irradiation controls (irradiated but not trypsinised) using the agar culture assay.

Results

There was no significant difference in GM-CFC survival between trypsinised cells irradiated at different time intervals following trypsinisation (F-test, $P < 0.05$).

Figure 3.13 depicts GM-CFC survival with increasing time between the two treatments.

Figure 3.13: Response of GM-CFC from (0.05 %) femoral bone marrow cells irradiated at diiferent time intervals with a single dose of X-rays (2 Gy) following trypsinisation (0.05 %). Results are mean ± SE from three plates in a single experiment.



3.3.7 Radiation responses of murine femoral bone marrow GM-CFC following trypsinisation.

Experimental procedure

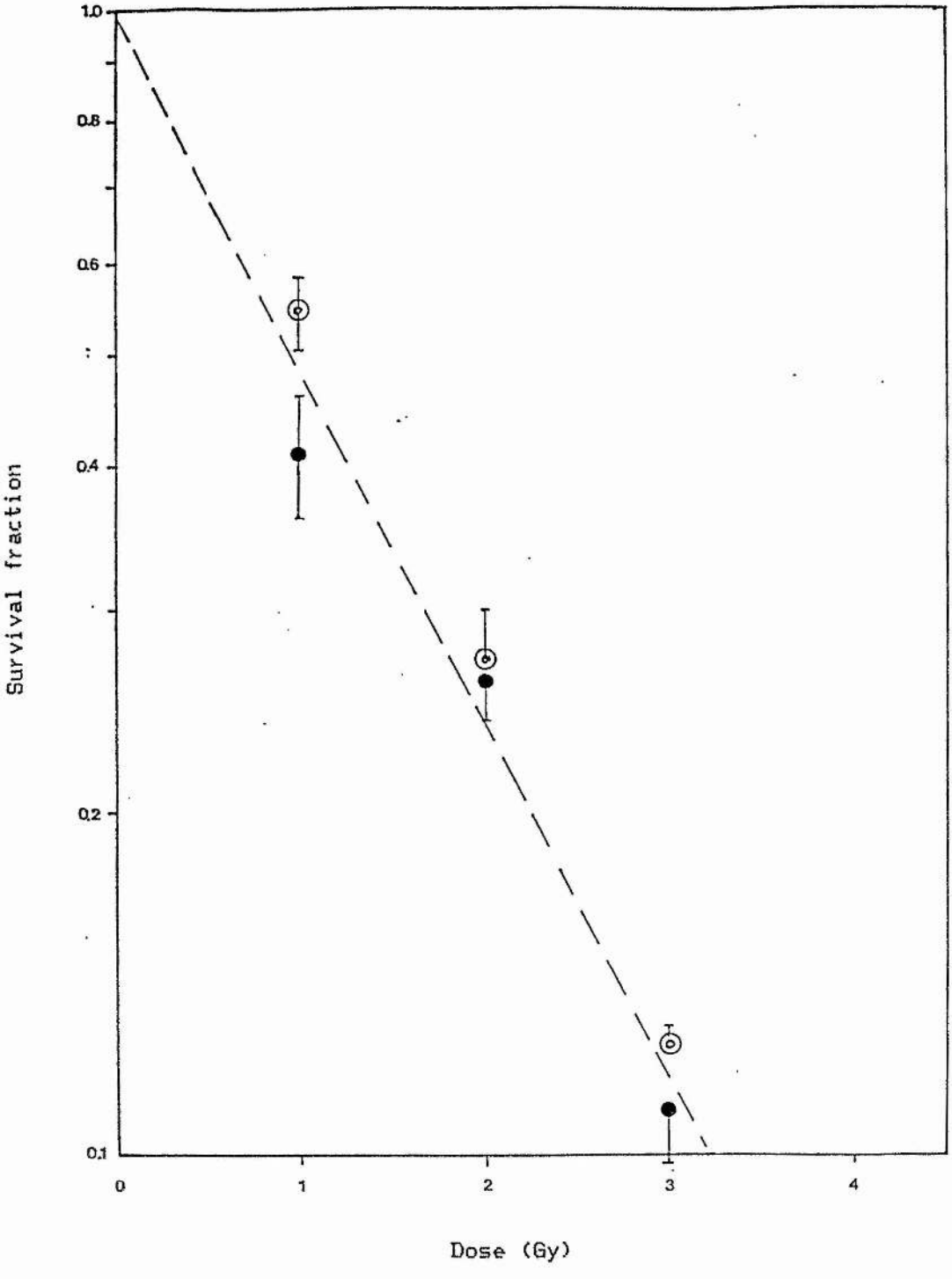
Pooled femoral bone marrow cells were trypsinised (10 mins., 37^{o}C) with 0.001% trypsin. The resulting pellet of cells were resuspended in serum-free Fischers medium. Untreated and pre-trypsinised cells from the same pooled suspension of marrow cells were irradiated with X-ray doses between 0 - 3 Gy. GM-CFC content was measured using the agar culture assay.

Results

The survival fractions were expressed as a fraction of the trypsin controls in the case of the irradiated pre-trypsinised cells and of the no treatment control for the irradiated cells not treated with trypsin.

A comparison of regression coefficients of the two dose-survival curves obtained (Figure 3.14) indicate no significant difference at $p < 0.05$. Trypsinised marrow cell GM-CFC show similar characteristic to untreated cells. A significant log-linear relationship is evident ($r > 0.999$, $P < 0.001$) and shows no evidence of a shoulder (Regression analysis, estimated value of $n = 1.00 \pm 0.01$). The estimated value of D_o was 1.20 ± 0.07 Gy.

Figure 3.14: Radiation survival curve of GM-CFC from trypsinised (⊙) and untreated (●) femoral bone marrow cells. Values are mean ± SE from five plates in a single experiment.



3.4 RESTRICTION ENZYME EXPERIMENTS

3.4.1 The survival response of pre-trypsinised femoral bone marrow GM-CFC to restriction endonucleases (Pvu II and Bam HI) and endonuclease storage buffer.

Experimental procedure

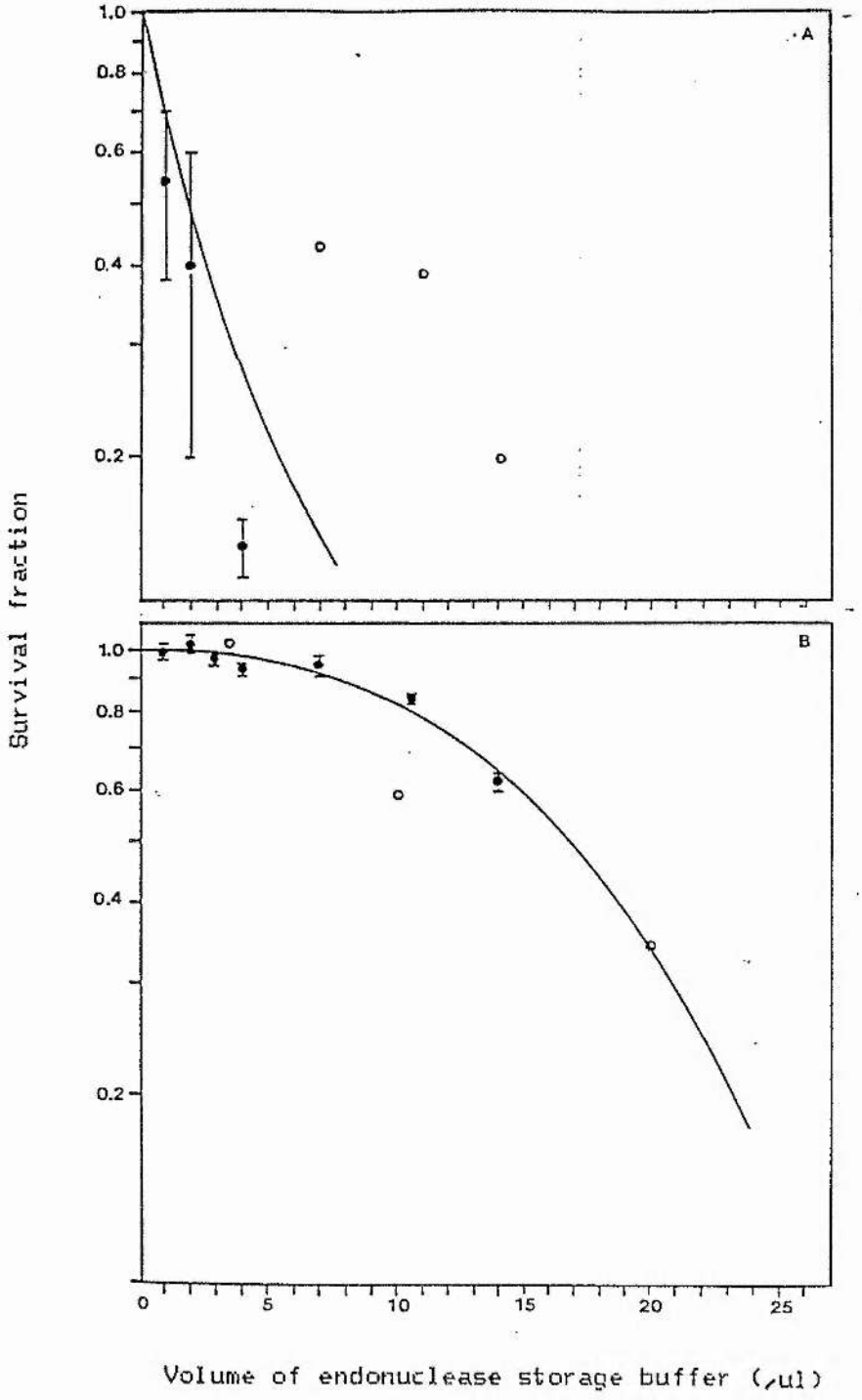
Normal femoral bone marrow cells were rendered permeable to restriction endonucleases using trypsin at concentrations of 0.05 and 0.001%. Restriction endonucleases (Pvu II and Bam HI) or storage buffer alone were subsequently added to the pre-trypsinised cells. The Pvu II activity added were between 0 to 200 units. In the Bam HI experiments, only cells pre-trypsinised with 0.001% trypsin were investigated. Cells were then assayed for their clonogenic ability using the GM-CFC agar culture assay.

Results

Figures 3.15a and 3.15b shows the effect of increasing amounts of storage buffer on GM-CFC survival following treatment with trypsin expressed as a fraction of the trypsin control. The survival of GM-CFC decreases with increasing amounts of storage buffer following pre-treatment with trypsin at both concentrations used. Cells pre-treated with 0.05% trypsin are more sensitive to the storage buffer showing a more rapid decline (Figure 3.15a). However, cells pre-treated with the lower trypsin concentration were less sensitive, a shoulder effect was significantly apparent with lower volumes or doses of storage buffer (F-test for data obtained between 1 - 4 μl of storage buffer showed no significant difference, $P > 0.1$).

Following these results, response curves obtained with restriction endonucleases were expressed as a fraction of

Figure 3.15: Effects of endonuclease storage buffer on GM-CFC from femoral bone marrow pre-treated with A) 0.05 % and B) 0.001 % trypsin. (●) are mean values ± SEM from five experiments and (○) are mean values of a single experiment.



corresponding storage buffer controls. Only values from experiments using less than 14 /ul of storage buffer containing restriction endonuclease were included in the data reported here ie. where the minimum survival fraction obtained in the storage buffer control was 0.62 $\pm$ 0.02.

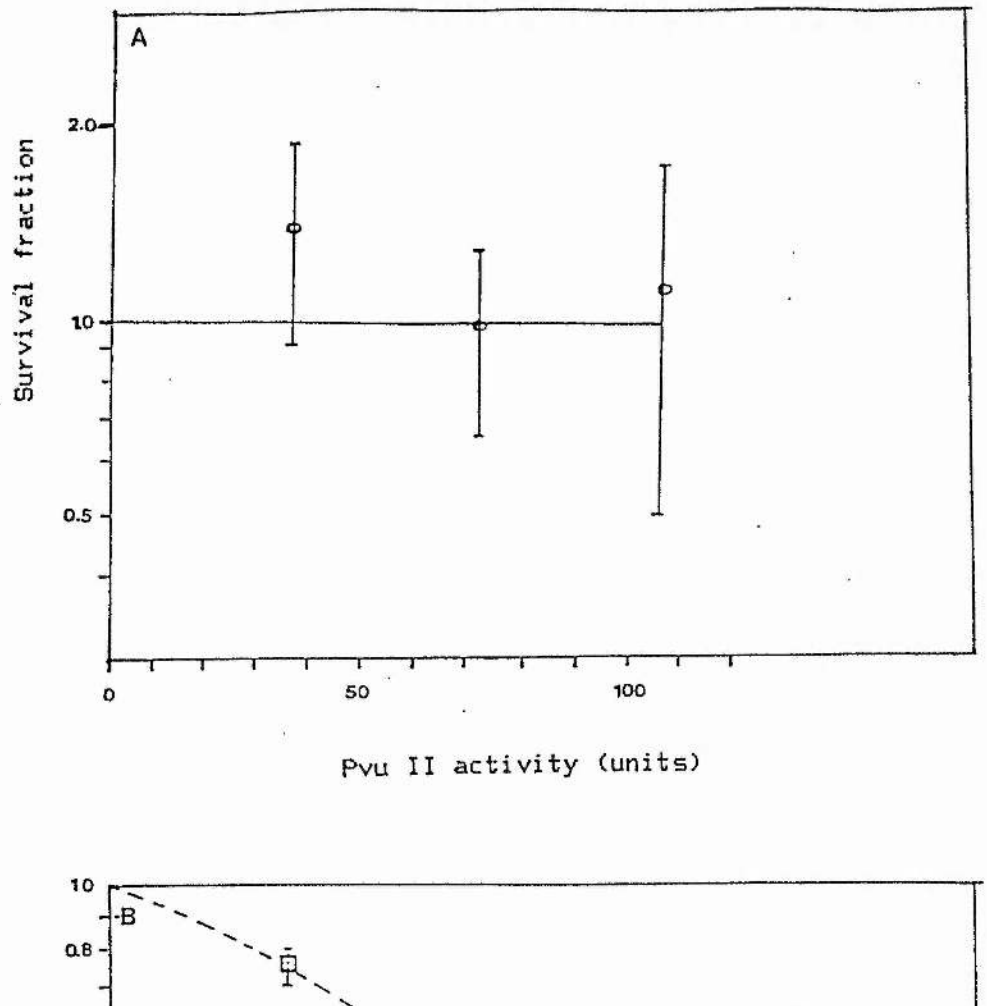
Figure 3.16a shows the survival fraction of GM-CFC with increasing activity of Pvu II following pre-treatment with 0.05% trypsin. There was no additional reduction in GM-CFC survival following addition of restriction endonuclease (F-test, $P > 0.1$) over the toxic effects of the storage buffer. Figure 3.16b illustrates the toxic effect on the survival of GM-CFC by the corresponding storage buffer controls expressed as a fraction of trypsin control (Pvu II activity = 35 units ml^{-1}).

The survival curves of GM-CFC with restriction endonucleases Pvu II and Bam HI following pre-treatment with 0.001% trypsin are shown in Figure 3.17 and 3.18 respectively. The effect of Pvu II was significantly different from the respective storage buffer control (T-test at all the doses, $P < 0.01$). Bam HI is ineffective in causing significant loss of clonogenic ability of GM-CFC even when higher activities than Pvu II were used. The survival fraction was not significantly different from the respective storage buffer controls (T-test at all the levels investigated, $P > 0.1$).

In Figure 3.19, the GM-CFC survival curve of marrow cells pre-treated with 0.001% trypsin (Figure 3.14) is included for comparison with the survival curves obtained with Pvu II and Bam HI. D_{37} value for Pvu II was estimated to be 142 $\pm$ 4 units and the relationship shows no apparent evidence of a shoulder (Regression analysis, estimated value of n was 0.99 $\pm$ 0.03).

Figure 3.16: Survival dose curve of GM-CFC from pre-trypsinised (0.05 %) femoral bone marrow cells treated with A) Pvu II and B) corresponding volumes of storage buffer.

Values are mean ± SEM of three experiments. Lines are fitted by eye to the survival fraction data only.



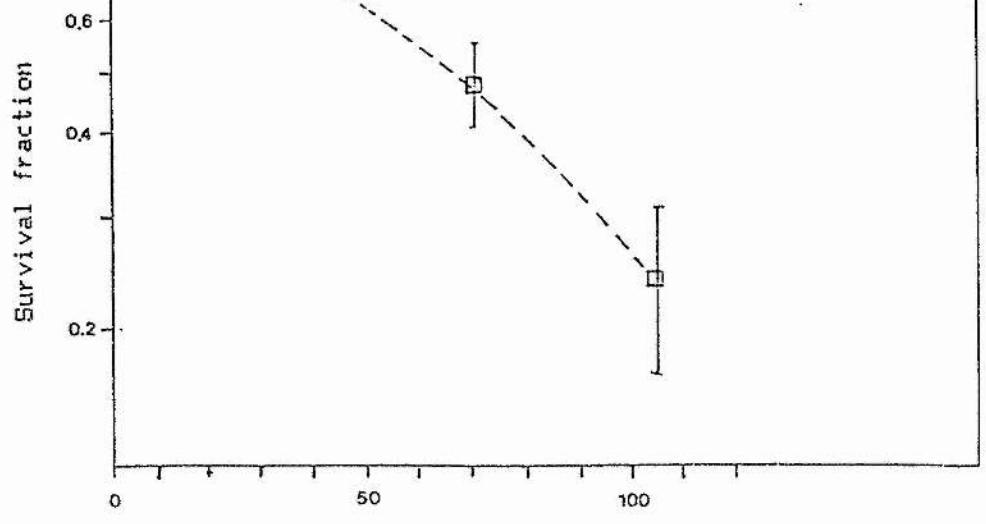


Figure 3.17: Survival curves of GM-CFC from pre-trypsinised (0.001 %) bone marrow cells treated with Pvu II restriction endonuclease and storage buffer. Values are means ± SEM of at least three experiments. A - Storage buffer controls, B - Pvu II tests.

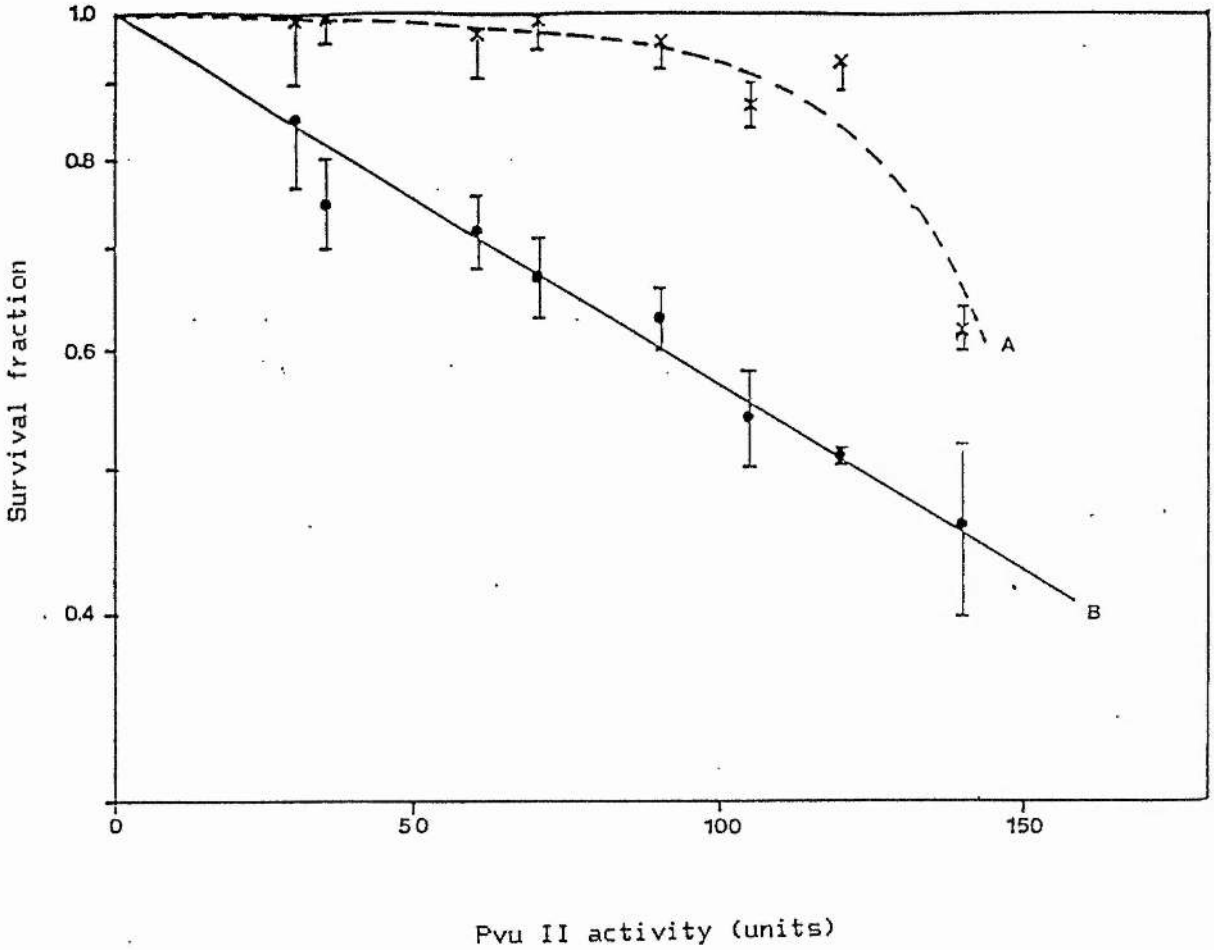


Figure 3.18: Survival curves of GM-CFC from pre-trypsinised (0.001 %) bone marrow cells treated with Bam HI restriction endonuclease and storage buffer. Values are means ± SEM of at least three experiments. A - Storage buffer controls, B - Bam HI tests.

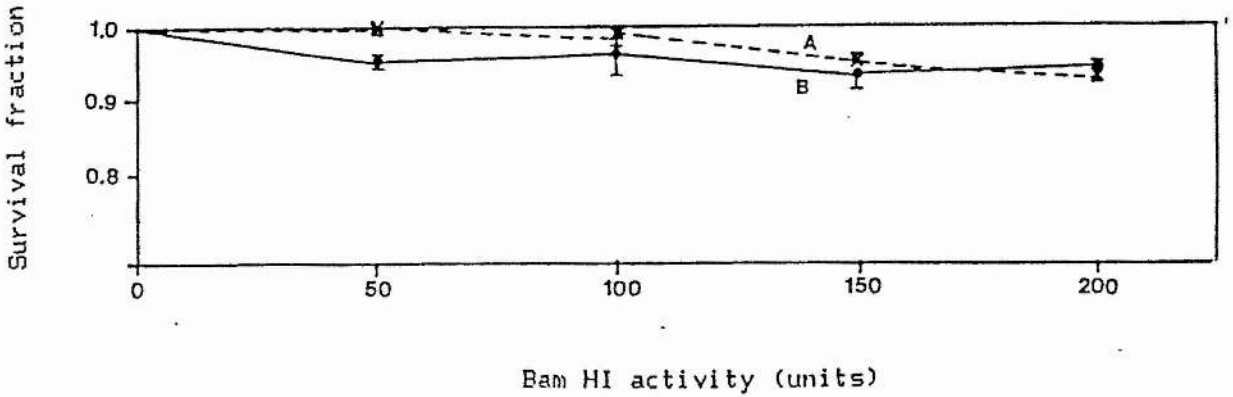
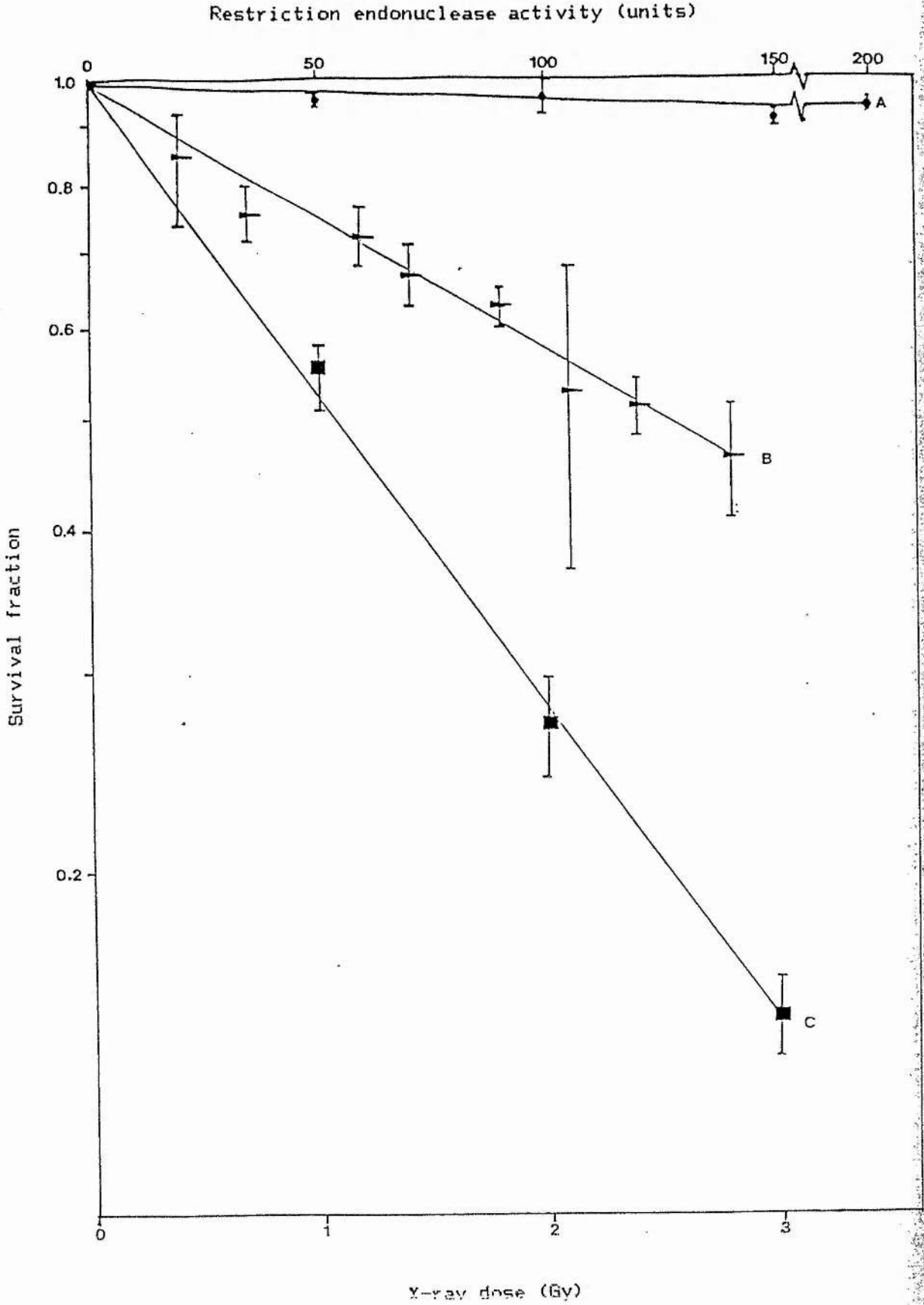


Figure 3.19: Survival curves of GM-CFC from pre-trypsinised (0.001 %) bone marrow cells treated with restriction endonucleases Pvu II and Bam HI compared to X-rays. Values are means ± SEM of at least three experiments. A - Bam HI, B - Pvu II and C - X-rays.



A comparison of the coefficient of regression of the survival curves for Pvu II and Bam HI showed significant difference between the curves obtained ($P < 0.01$).

4.0 DISCUSSION

CHAPTER FOUR

DISCUSSION

There are remarkably few papers in the literature where the results of clonogenic and non-clonogenic assays have been compared in well-characterised experimental systems. Generally, isotope uptake assays can be divided into short-term assays which measure the effect of cytotoxic insult on specific biochemical processes (eg. DNA or protein synthesis) within a very few hours of removal from the organ/tissue (Volm et al., 1979; Sanfilippo et al., 1981) and those assays which depend on the rate of proliferation of cells established in culture some days after the removal and treatment. In the following discussion, we will only consider the latter type of assay as it is clearly more akin to a clonogenic assay but performed at an earlier stage of growth.

To date the most promising method for assesing both drug and radiation sensitivity of bone marrow GM-CFC has been the measurement of the ability of the cytotoxic agents to kill those cells which are capable of cloning in vitro. With respect to assesing the effect of chemotherapeutic drugs to leukaemic cells, it is not clear as to whether the utility of the clonal assays derives from the fact that the effects of drugs on a 'relevant' subpopulation of leukaemic cells are being measured or if it is simply due to the fact killing per se is being assesed (Epstein & Preisler, 1983).

Most studies examine the effect of cytotoxic drugs on the clonogenicity and ^{3}HTdR incorporation of various mammalian (normal or tumour) cell lines. Earlier studies (Roper & Derwinko, 1976; Rupniak et al., 1983) had shown that the results did not correlate and it was found not possible to define a quantitative relationship between the two assays. Recent studies have shown that the results correlated very well (Horak et al., 1983; Epstein & Preisler, 1983; O'Brien & Horton, 1984, using ^{3}H-galactose).

Friedman et al. (1983) obtained dose-effect curves for the radiation response of CHO cell line and the results were found to be closely similar to those obtained with the clonogenic assay and this were in agreement with results found by Twentyman et al. (1984).

The studies presented here provide the radiation survival curve for mouse femoral bone marrow GM-CFC for both the clonogenic agar culture assay and the ^{125}IUdR or ^{3}HTdR uptake assay. The uptake assay employing either ^{125}IUdR or ^{3}HTdR were assumed to be similar (A.C. Riches, personal communication).

When the differences in irradiation and culture conditions, assay times and procedures are taken into consideration, the D_{37} and extrapolation number (n) obtained with mouse femoral bone GM-CFC using the agar culture assay generally agree with those in literature (see Table 1.5).

More recently, a review of femoral GM-CFC in mouse estimates that the mean D_o for X- rays was 1.79 $\pm$ 0.10 Gy for 4 mesurements. The review included those irradiated in vitro and in vivo, and concluded that there was no shoulder to the survival curve. Included in the review was one measurement with X-rays

(Wilson et al., 1974) which showed a much lower value of D_o of 0.7 Gy similar to that of CFU-S and the review associated this anomaly to plating efficiency. A large RBE between x-rays and gamma-rays is not expected since the cycling GM-CFC shows an RBE near to unity (Hendry & Lord, 1983).

We have attempted to estimate the mean survival curve parameters of the data shown in Table 1.5. For GM-CFC in the mouse the mean D_o for x-rays is 1.61 $\pm$ 0.11 Gy for 11 measurements (including those calculated by Hendry & Lord, 1983) and there is no shoulder (value of n approximates unity ie. 0.96 $\pm$ 0.05).

The present data demonstrate that mouse femoral bone GM-CFC sensitivity were generally similar when irradiated either in vivo or in vitro. This was true for both the agar colony assay or the radioisotope assay and agrees with earlier findings (Lin, 1975).

It is stressed that the results are presented in terms of clonogenic units (GM-CFC) per 10^6 nucleated cells plated. For this reason, it was important to examine the data of the clonogenic assay in terms of absolute number of GM-CFC present in marrow populations. Presented in this way, the data do show significant similarities in response to increasing doses of radiation.

The radiation survival curves obtained by the radioisotope assay have a highly significant correlation with the clonogenic assay (Linear correlation coefficient, $P < 0.001$) and also showing no evidence of a shoulder. However, it also shows significant increase in the D_{37} value as compared to the clogenic assay, thus indicating reduction in radiation sensitivity.

In both the assay systems, colony stimulating activity is required to stimulate the proliferation and/or the formation of colonies. Although the two assays show good correlation, the D_{37} value proves that the two assays are not measuring identical cellular events.

Whereas the clonogenic assay will measure only the long term survival of cells by virtue of the definition of the term viable cells viz. colonies that contain 50 or more cells, the radioisotope uptake assay will additionally reflect the various lengths of delay induced by the radiation and also any changes in subsequent proliferation rate of surviving cells. If the colony size is reduced by a given treatment, this factor will not be accounted for in a clonognic assay but would produce a reduced radioisotope uptake. Hence, unlike the clonogenic assay, the radioisotope assay reflects an integral effect of the cytotoxic insult.

A 2-day delay between irradiation exposure and the addition of ^{125}IUdR or ^{3}HTdR has been used to accomplish allowance of any transient inhibitory effects of radiation upon macromolecular synthesis to dessipate and to permit enough time to elapse so that irreversibly damaged cells which might be able to go through one cell division after exposure would have done so before the addition of the radioisotope label. By setting these conditions, it is likely that ^{3}HTdR or ^{125}IUdR incorporation is a measure of unaffected proliferating cells in the culture and these permit the measurement of of the total number of the cells which have survived and are proliferating under the influence of colony stimulating factor.

The radioisotope uptake assay has the advantage of functioning even when clonal growth is not apparent in systems which utilise a semi-solid matrix. An example, is the determination of the surviving fraction of GM-CFC to higher doses of irradiation (> 8 Gy of X-rays). It becomes extremely difficult to resolve colonies in a background of a high density of cells that were seeded to account for the high killing effect at higher doses in order to achieve statistically reliable results. The assay is also simple to use, experiments are rapidly and objectively evaluated and the data is available in half the time that the colony assay systems require. The increasing technology and sophistication behind both the Gamma- and Beta-counters will additionally contribute to objective and consistent evaluation that at present is subjective in the colony assays and prone to human errors.

The assay, too, has its problems. It suffers from the disadvantage of being indirect compared to the agar culture assay for GM-CFC, which measures GM-progenitor cells growth directly. Secondly, the specificity of the assay remains to be proven conclusively. The assay may reflect more accurately if the uptake response to CSA used in myeloid progenitor cells demonstrate similar efects when pure CSA is used in combination with a population of homogenous target cells as provided by a cell line or prefractionation of the progenitor cell population using techniques such as fluorescence-activated cell sorting (FACS) either on the basis of single light scatter or coupled with fluorescein-conjugated probes (for review see Nicola, 1982). Lastly, because of the pyrimidal system involved in haemopoietic cell proliferation, ^{3}HTdR or ^{125}IUdR uptake assay is likely to

reflect activity of myeloid cluster-forming cells and possibly cells that are even more differentiated. Thus, all these factors may reflect the reduction in X-ray sensitivity using the radioisotope uptake assay.

In the agar culture assay, it is evident that at low concentrations of CSA, a high proportion of only macrophage colonies develop, whereas at higher concentrations, the proportion granulocyte-containing colonies are stimulated to develop in mouse femoral bone marrow cells. These are consistent with the findings reviewed in Metcalf (1984).

We report here that although the effect of X-rays on the proliferative capacity of GM-CFC can be recovered by the increasing concentrations of CSA in the agar culture assay, (as suggested by Sugavara et al., 1980) the recovery is not continous.

At the level of concentration of CSA used in the agar culture assay, it is evident that further increase in concentration does not further change the number of GM-CFC affected by X-rays. Therefore, the term recovery to describe the of effect of increasing numbers of GM-CFC with increasing CSA at low concentrations is misleading. When conditions are optimised, the agar culture assay would manifest all the unaffected GM-CFC.

It was noted that the increase of cluster-colony ratio was more prominent in the culture containing lesser amounts of CSA. Yet, it was apparent that the increase in the number of colonies was not accountable by the decrease in the number of clusters suggesting that the two are distinct populations which may be related. It becomes evident that the potency of CSA used to

stimulate cultures of irradiated cells modify the D_o value obtained and further stresses the importance of optimising the amounts of CSA added in an assay.

In the present investigations, no endogenous CSA was detected in either the treated or the untreated femoral bone marrow cells. The variations in reported result of D_o of mouse femoral bone marrow GM-CFC of 0.7 Gy (Wilson et al., 1974) to 2.37 Gy (Wu et al., 1978) may be due to differences in the conditions of irradiation or in the time between exposure and sampling of marrow; to list but a few. The colony stimulating system adopted cannot be discounted in this respect when lower D_o is achieved. Further sources of variation may be related to the different strains of mice used, or to the differences in details of the in vitro culture conditions. The data reported is comparable to the radiation sensitivity of GM-CFC derived from the human manubrium with increasing CSA (Broxmeyer et al., 1976) and indicates that low Do values are obtained when cultures are stimulated with CSA of low potency. Clearly, although the number of colonies formed is a function of the amount of the CSA present, log dose irradiation survival curves plated with optimum or higher potency of CSA should parallel one another and their value of D_o should be identical.

With respect to split acute doses, mouse femoral bone marrow GM-CFC showed a survival ratio oscillating with time as has been shown by Briganti & Mauro (1979). This feature has been attributed to phase sensitivity changes after the first dose (Hendry & Lord, 1983). The lack of a shoulder in the GM-CFC survival curve and the asynchronous nature of the normal bone marrow cells makes it difficult to characterise the relationship

as the Elkind-type recovery curve (Hall, 1978) as no repair of sublethal damage can be attributed to it since sublethal damage in the population is not apparent.

The work on the effect of pre-trypsinisation of mouse femoral bone marrow has demonstrated that there are two subpopulations of granulocyte-macrophage progenitor cells: one which is sensitive and dependent on trypsin concentration and another which is entirely unaffected by trypsin even at higher concentrations.

Recovery experiments post-trypsinisation have shown that GM-CFC survival does not recover with time nor does it further reduce. The fact that untreated normal cells show similar features infer that should damage by trypsin be incurred on GM-CFC, no repair mechanisms seems to occur in vitro.

In our effort to identify the trypsin sensitive subpopulation in femoral bone marrow GM-CFC, our results show that the effect of trypsin and ARA-C (an S-phase specific drug) are independent regardless of the initial pre-treatment used with either agents. Comparing the synergistic effect of both the trypsin and ARA-C, the data suggests that the femoral bone marrow cells treated initially with ARA-C and followed by trypsin shows an increase in sensitivity although the proportion of cells killed by trypsin remains essentially the same. This has no significance as the expected survival fraction from the treatment of ARA-C and trypsin is not significantly different with either treatments.

We conclude that the effect of trypsin on GM-CFC is not selective in particular on GM-CFC in DNA-synthesis, rather the

effect is indiscriminate and in a random manner. It is possible that these trypsin-sensitive cells are in the other stages of the cell cycle but in the scope of this report we are unable to draw any conclusions.

The proportion of mouse femoral bone marrow GM-CFC in DNA-synthesis (S-phase) determined in this study was comparable to that of other findings (Cork et al., 1982; Broxymeyer et al., 1976).

Further studies on the effect of irradiation of single doses of X-rays at different times following trypsinisation (0.05% trypsin) show no significant change in resistance by GM-CFC over 6 hours. We are unable to demonstrate the resistance observed by other workers (Bryant & Parker, 1979; Barendsen & Walter, 1964; Philips & Tolmach, 1964; Berry et al., 1966). It was postulated that following trypsinisation, the cell population is partially synchronised, with consequent redistribution of cells among the various sensitivity states that are characteristic of different periods of cell division cycle.

The radiation survival curve obtained for mouse femoral bone marrow GM-CFC immediately after trypsinisation (0.001% trypsin) of cells is not significantly different from the radiation sensitivity of GM-CFC from untreated bone marrow cells. The work of Philip & Tolmach (1964) and Berry et al. (1966), using 0.03 and 0.1% trypsin solutions respectively, report a transient drop in sensitivity to X-rays immediately after trypsinisation followed by an increase.

It was shown previously that Chinese hamster cells permeabilised with in activated Sendai virus and treated with restriction endonuclease Pvu II causes 'pure' double strand

breaks in DNA (Bryant, 1984). In the absence of other forms of damage, these lesions were found to have led to the formation of chromosomal aberrations (Bryant,1984; Natarajan & Obe, 1984). Bender et al. (1974) proposed that final expression of polynucleotide strand breaks as chromosomal aberrations is the consequence of the operation of the repair mechanisms, DNA replications and single strand (ss) DNA'ases. It was postulated that the blunt-ended dsb induced by Pvu II are sometimes left misrepaired, or left un repaired by the cell, leading to exchange and deletion types of chromosomal abberations. Lethality as the consequence of the induction of chromosomal aberrations by the endonuclease Pvu II has been shown to lead to the loss of clonogenic ability (Bryant, 1985).

Clearly, our data show that when normal bone marrow cells were permeabilised by 0.001% trypsin, the blunt-ended dsb thought to be induced by the introduction of of Pvu II led to the loss clonogenic ability of GM-CFC. It has been demonstrated by Bryant (1985) that this lethality is linked with the induction of chromosomal aberrations. A corresponding lack of killing effect on GM-CFC was also observed for Bam HI on bone marrow cells permeablised at the same trypsin concentration. The work of Bryant (1984) had shown that Bam HI was inducing dsb at the same frequency as that for Pvu II but it did not yield a chromosomal aberration frequency as would be expected and he attributed it to the ability of the cells to repair this type of lesion efficiently. This conforms to the model that cell killing in the case of Pvu II results from the induction of chromosomal aberrations.

Ionising radiations of low LET produce single- and double-stranded breaks in the ratio of 20:1 (Bender et al., 1974) and in addition a variety of other probable lesions occur. Amongst others, these may be sugar or in particular, base lesions, capable of leading to chromosomal aberrations (Preston, 1980). Endogenous cellular endonuclease has been proposed to cause base damage leading to the shearing of DNA in mammalian cells ie. dsb (Ahnstrom & Bryant, 1982).

The work presented here using restriction endonuclease treatment on permeabilised bone marrow cells to a certain extent simulates the exposure of cells to X-rays by inducing blunt-type dsb in DNA in GM-CFC without the complicating presence of other types of lesions. Based on the previous simulation by the use of Pvu II (Bryant, 1985), it is not impossible to speculate that X-irradiated cells undergo a mode of cell death in which DNA dsb induced by X-rays lead to GM-CFC mortality via the formation of chromosomal aberrations through the loss of genetic material, misrepair or dsb being left unrepaired. However, we are unable to confirm this as no chromosomal work was performed.

In contrast to previous work on the use of endonucleases (Natarajan & Obe, 1984; Bryant, 1984 & 1985;) this work attempts to introduce the use of trypsin to permeablise cells for the treatment with endonuclease and also the possibility of generating blunt-type dsb in GM-CFC from mouse femoral bone marrow cells with Pvu II without the use of a non-viral permeabilising agent.

The use of femoral bone marrow GM-CFC presents a realistic model of damage of normal tissues by X-rays; normal tissues being of a heterogenous population of cells. More importantly, it is

plausible that Pvu II sites do exist in bone marrow GM-CFC and its scission has led to the loss of clonogenic ability in the clonogenic assay. Hence, Pvu II is potentially clastogenic or lethal, in relation to cell killing of GM-CFC of mouse bone marrow cells.

Storage buffer in which the restriction endonucleases are contained was shown to have a lethal effect on the survival of GM-CFC from pre-trypsinised bone marrow cells. The presence of an initial shoulder in the survival of GM-CFC from bone marrow cells pre-trypsinised with the lower concentration of trypsin used (0.001%) indicates that there was a definite tolerance of GM-CFC to the toxic effects of the storage buffer. Provided that the restriction endonucleases to be investigated is contained in a volume lying in the shoulder region, the true effects of the endonucleases may be investigated. It is neither possible at this stage to identify the chemical(s) or the physical parameters in the storage buffer responsible for its killing effects of GM-CFC nor to speculate if the cells were able to repair the lesions caused by the storage buffer. However, it is worthy to note that cells trypsinised with the higher trypsin concentration (0.05%) shows no region of tolerance to the toxicity of the storage buffer but show a more rapid increase in sensitivity. There may be two possible mechanisms to this. Trypsin may have apparently sensitised the sites which were sensitive to the storage buffer or that there were residual trypsin not completely neutralised by the addition of serum. In the latter, the subsequent incubation period would have further aggrovated the damage in the trypsin sensitive sites. This cannot be the case as investigations show

that the effect of trypsin was not incubation time dependent, therefore this second mechanism was not likely to be true. Although this rationale is based on incubation time dependence studies investigated to a maximum of 10 mins., it is further pointed out that the addition of adequate quantities of horse serum was believed to have been sufficient to have curbed residual trypsin activity. It is likely that the mechanism was the synergistic effect of trypsin and the storage buffer. Based on these findings, the effects of restriction endonuclease on GM-CFC from cells permeabilised with 0.05% trypsin was discontinued and the volume of storage buffer containing the endonuclease was restricted to 14 /ul. This was adopted so as not to complicate damage caused by either trypsin at a higher concentration or the toxic properties of the storage buffer. Moreover, a large number of cells would have to be plated so as to account for the killing effect of the trypsin and the storage buffer. This would have resulted in difficulty in defining the colonies produced during assay using the agar culture technique.

Cell permeabilisation by the use of 0.001% trypsin was successful by the results obtained for the Pvu II experiments. GM-CFC survival was dose dependent to Pvu II and provides conclusive evidence that restriction endonucleases were able to enter the cell to subsequently cause loss of clonogenic ability. The absence of further damage by Pvu II to GM-CFC from femoral bone marrow permeabilised with 0.05% trypsin suggest that specific sites of action of Pvu II were absent or that such blunt-type lesions would have no synergistic effect over the damage already incurred by the treatment of storage buffer and trypsin. The presence of DNA'ases in the trypsin used cannot be

excluded.

Bryant (1984) suggested that 100 units of Pvu II was equivalent to 11 Gy of X-rays on the basis of their ability to produce the same amount of dsb in DNA strand break measurements. Using DNA unwinding techniques, it was also estimated that 500 units of Pvu II yielded an unwinding value equivalent to 2 Gy of X-rays comprising of single- and double-strand breaks. Based on these facts it was inferred that dsb induced by Pvu II were relatively inefficient in causing detectable aberrations compared to X-rays.

This work suggests that an equivalent dose of Pvu II to the dose required to reduce the number of GM-CFC to 37% of their former value ie. D_{37} was 142 $\pm$ 4 units of Pvu II and this was equivalent to 1.20 Gy of X-rays ie. value of D_o for GM-CFC from pre-trypsinised bone marrow cells or 100 Pvu II units = 0.85 Gy. This value takes into account damages due to single- and double-strand breaks. For a line of V79 Chinese hamster cells, Bryant (1984), 100 units of Pvu II was found to be approximately equivalent to 11 Gy of X-rays, based on the dose which would have to induce more than one aberration per cell.

This report here has provided a cheaper and more economical alternative to inactivated Sendai virus to permeabilise mammalian cells in introducing restricted endonucleases to study damage incurred by irradiation. Moreover, where aseptic techniques are involved, the use of non-viral agents such as trypsin proves to more advantageous. However, it is worth confirming the efficiency of the permeabilising procedure, possibly by the use of dyes and also investigating as to whether the use of trypsin does affect

the integrity of the DNA of the cell to be investigated. The importance of having demonstrated two subpopulations of GM-CFC based on their sensitivity to trypsin is not yet apparent but future work in using trypsin on mouse femoral bone GM-CFC must bear this fact into consideration when determining the response of pretrypsinised bone marrow cells.

The work in determining the frequecy of the different types of chromosomal aberrations formed by the use of Pvu II and Bam HI in mouse femoral bone GM-CFC is worth considering so as to confirm the relation of loss of clonogenic ability to dsb as suggested by this report.

It is also worth investigating as to whether the use of restriction endonucleases can provide a possible model to study the induction of myeloid leukaemia in the mouse by X-rays. To date, there are over 55 restriction endonucleases available commercially, the task of simulating such a phenomena is enormous and may or may not involve the use of several restriction endonucleases in combination or in series.

REFERENCES

Alberts B., Bray D., Lewis J., Raff M., Roberts K., and Watson J.D. (1983). Molecular Biology Of The Cell. Garland Publishing; New York, pp 177

Aherne W.A, Camplejohn R.S. and Wright N.A. (1977). An Introduction To Cell Population Kinetics. Edward Arnold; London, pp 58

Ahnstrom G. and Bryant P. (1982). DNA double-strand breaks generated by the repair of X-ray damage in Chinese hamster cells. Int. J. Radiat. Biol., 41: 671

Aizawa S., Amaki I., Miyanome T., Tsurusawa M. and Mori K.J. (1984). Survival and repopulation of irradiated 'pre-CFU-C' in mice. Cell. Tissue Kinet., 17: 142

Barendsen G.W. and Walter H.M.D. (1964). Effects off different ionising radiations on human cells in tissue culture: IV Modification of radiation damage. Radiat. Res., 21: 314

Bender M.A., Griggs H.G. and Bedford J.S. (1974). Mechanisms of chromosomal aberration production: III. Chemicals and ionising radiation. Mutation Res., 23: 197

Berry R.J., Evans H.J. and Robinson D.M. (1966). Perturbations in X-ray dose response in vitro with time after plating: A pitfall in the comparison of results obtained by different laboratories using asynchronous cell systems. Exp. Cell. Res., 42: 512-522

Boehringer Manhiem GmbH, Biochemica (1985). Biochemicals For Molecular Biology. Gebr Parcuss; Munich

Bosanquet A.G., Bird M.C., Price W.J.P. and Gilby E.D. (1983). An assesment of a short-term tumour chemosensitivity assay in chronic lymphocytic leukaemia. Brit. J. Cancer. 47: 781

Bradley T.R. and Metcalf D. (1966). The growth of mouse bone marrow in vitro. Aust. J. Exp. Biol Med. Sci., 44: 287

Briganti G. and Mauro F. (1979). Differences in radiation sensitivity in subpopulations of mammalian multicellular systems. Int. J. Radiat. Oncol. Biol. Physics, 5: 1095

Broxymeyer H.E., Galbraith P.R. and Fraser L.B. (1976). Relationship of colony-stimulating activity to apparent kill of human colony-forming cells by irradiation and hydroxyurea. Blood, 47: 403

Bryant P.E. (1984). Enzymatic restriction of mammalian cell DNA using Pvu II and Bam HI: Evidence for the double-strand break origin of chromosomal aberrations. Int. J. Radiat. Biol., 46: 57

Bryant P.E. (1985). Enzymatic restriction of mammalian DNA: evidence for double-strand breaks as potentially lethal lesions. Int J. Radiat. Biol., 48: 55

Bryant P.E. and Parker J. (1979). Changes in sensitivity of HeLa cells after split doses of X-rays. Int. J. Radiat. Biol., 35: 189

Burgess A.W. and Metcalf D. (1980). The nature and action of granulocyte-macrophage colony stimulating factors. Blood, 56: 947

Chen M.G. and Schooley J.C. (1968). A study on the clonal nature of the spleen using chromosome marker. Transplantation, 6: 121

Carrano A.V. (1973). Chromosomal aberrations and radiation induced cell death: II Predicted and observed cell survival. Mutation Res., 17: 355

Coggle J.E. (1977). Biologial Effects of Radiation. The Wykeham Science Series (Mott, N., Noakes, G.R. and Yapp, W.B. eds.). Wykeham; London

Cork M.J., Wright E.G. and Riches A.C. (1982). Regulation of the murine granulocyte-macrophage progenitor cells and haemopoietic stem cell proliferation by factors produced in the human foetal liver. Leukaemia Res., 6: 553

Dethlefsen L.A. (1970). Reutilisation of ^{131}I-5-iodo-2'-deoxyuridine as compared to ^{3}H-thymidine in mouse duodenum and mammary tumour. J. Natl. Cancer Inst., 6: 173

Dethlefsen L.A. and Riley R.M. (1973). Hydroxyurea effects in C3H mouse. II Mammary cell kinetics. Cell Tissue Kinet., 6: 173

Dewey W.C., Miller H.H. and Leeper D.B. (1971). Chromosomal aberrations and mortality of X-irradiated mammalian cells: Emphasis on repair. Proc. Natl. Acad. Sci., U.S.A., 68: 667

Dexter T.M. and Testa N.G. (1976). Differentiation and proliferation of haemopoietic cells in culture. In Methods In Cell Biology (Prescott D. ed.). Academic Press, New York, pp 387

Domon M. (1980). A biological variability model of cell survival curves. Radiat. Res., 82: 611

Epstein J. and Priesler H.D. (1983). A non-clonogenic assay for the determination of the sensitivity of leukaemic cells to chemotherapeutic agents. Eur. J. Clin. Oncol., 19: 1569

Fox M. (1986). Assay of colony-forming ability in established cell lines. In Cell Clones: Manual of mammalian cell techniques (Potten C.S. and Hendry J.H. eds). Churchill Livingstone; London, Edinburgh, pp 184

Frankenberg D., Frankenberg-Schwager M.and Harbich R. (1984). Interpretation of the shapes of survival curves in terms of induction and repair/misrepair of DNA double-strand breaks. Proc. 11th L.H. Gray Conference, Glasgow, July, 1983. Brit. J. Cancer Suppl VI, 49: 223

Friedman H.M., Candilis P., Johnson G. and Glaubiger D.L. (1983). In vitro radiation survival curves from human tumour cell lines and primary human tumour explants using (^{3}H)thymidine assay. Proc. AACR, 1983, pp 316

Golde W.D. and Cline M.J. (1974). Regulation of granulopoiesis. New Eng. J. Med., 281: 1388

Gordon M.Y. (1975). A comparison of the radiosensitivity and o.e.r. of human and mouse progenitor cells cultured in agar in diffusion chambers. Int. J. Radiat. Biol., 28: 285

Grote S.J. and Revell S.H. (1972). Correlation of chromosome damage annd colony-forming ability in the Syrian hamster cells growing in culture irradiated in GI. Current Topics in Rad. Res. Q., 7: 303

Hall E.J (1972). Radiation dose rate: a factor of importance in radiobiology and radiotherapy. Br. J. Radiol, 45: 81

Hall E.J. (1978). Radiobiology For The Biologist. Harper and Rowe; Phliadelphia, Cambridge. pp 31

Hellman S., Grate E., Chaffey J.T. and Carmel R. (1970). Hematopoietic stem cell compartment: Patterns of differentiation following radiation and cyclophosphamide. In Haemopoitic Cellular Proliferation (Stohlman B. (Jr.) ed.). Grune and Stratton; New York, pp 37

Hendry J.H. (1985). Review: Survival curves for normal tissue clonogens: a comparison of assesments using in vitro, tranplantation, or in situ techniques. Int. J. Radiat. Biol., 47: 3

Hendry J.H. and Lord B.I. (1983). The analysis of the early and late response to cytotoxic insults in the haemopoietic cell hierarchy. In Cytotoxic Insult To Tissue: Effects on cell lineages (Potten C.S. and Hendry J.H. eds.). Churchill Livingstone; Edinburgh, pp 1

Horak H., Turner A.R., Shaw A.R.E. and Yau O.W. (1983). Stimulation of (^{3}H)Thymidine uptake in mouse marrow by granulocyte-macrophage colony stimulating factor from mouse lung conditioned medium. J. Immunol. Methods, 56: 253

Howard A. and Pelc S.R. (1953). Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity Suppl., 6: 261

Iscove N.N., Guilbert L.S. and Wayman C. (1980). Complete replacement of serum in primary cultures of erythropoietin dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acids, lecithin and cholesterol. Exp. Cell Res., 126: 121

Johnson G.R. and Metcalf D. (1977). Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythropoietin. Proc. Natl. Acad. Sci. U.S.A, 74: 3879

Joshi G.P., Nelson W.J., Revell S.H. and Shaw C.A. (1982). X-ray induced chromosomal damage in live mammalian cells and improved measurements of its effects on their colony forming ability. Int. J. Radiat. Biol., 41: 161

Kellerer M. (1975). Statistical and biophysical aspects of the survival curve. In Cell Survival After Low Doses Of Radiation: Theoretical and clinical applications (Alper T. ed.). Institute of Physics/Wiley; London, pp 69

Kellerer M. and Rossi H.H. (1972). The theory of dual radiation action. Curr. Top. Radiat. Res. Q., 8: 85

Lajtha L.G. and Oliver R. (1961). Some radiobiological considerations in radiotherapy. Br. J. Radiol., 34: 252

Lehnert S. (1975). Changes in radiosensitivity of V-79 cells accompanying growth and cell division. Radiat. Res., 63: 326

Lea D.E. (1955). Actions Of Radiations On Living Cells. Cambridge University Press; Cambridge, London, New York

Lin H.S. (1975). Peritoneal exudate cells: III Effect of Gamma-irradiation on mouse peritoneal colony-forming cells. Radiat. Res., 63: 560

Lloyd D.C., Purnott R.J., Dolphin G.W., Bolton D. and Edwards A.A. (1975). The relationship between chromosome aberrations and low L.E.T. radiation dose to human lymphocytes. Int. J. Radiat. Biol., 28: 75

Magli M.G., Iscove N.N., Ordartchenco N. (1982). The transient nature of early haemopoietic spleen colonies. Nature, 295: 527

Marsh J.C. (1976). The effects of cancer chemotherapeutic agents on hematopoietic precursor cells: A review. Cancer Res., 36: 1853

Metcalf D. (1977). Hematopoietic Colonies. Springer-Verlag; New York, pp 227

Metcalf D. (1984). The hemopoietic colony stimulating factors. Elsevier; Amsterdam, New York, Oxford.

Metcalf D. (1986). Review: The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. Blood, 67: 257

Metcalf D. and Moore M.A.S (1971). Haemopoietic Cells. Frontiers of Biology Volume 24. (Neurembeger A. and Tatum E.L. eds.). North Holland Publishing Company; Amsterdam, London

Metcalf D. and Moore M.A.S. (1973). Regulation and growth in haemotopoietic colonies growing in agar. In Haemopoietic Stem Cells. CIBA Foundation Symp. (Wolstenholme G.E.W. ed). Assosciated Scientific Publishers, 13: 157

Metcalf D., Johnson G.R. and Mandel T.E. (1979). Colony formation in agar by multipotential hemopoietic cells. J. Cell. Physiol., 19: 401

Millard R.E. and Blackett N.M. (1981). Radiosensitivity and recovery of two murine haemopoietic progenitor cell populations following gamma-rays and neutrons. Acta haemat., 66: 226

Nakahata T. and Ogawa M. (1982). Identification of in culture of a class of haemopoietic coloniy-forming units with extensive capacity to self-renew and generate multipotential haemopoietic colonies. Proc. Natl. Acad. Sci. U.S.A., 79: 3843

Natarajan A.T. and Obe G. (1984). Molecular mechanisms involved in the production of chromosomal aberrations. Chromosoma (Berlin), 90: 120

Natarajan A.T., Obe G., van Zeeland A.A, Palitti F., Meiyers M. and Vandegaal-Immerzeel E.A.M. (1980). Molecular mechanisms involved in the production of chromosomal aberrations. II Utilisation of Neurospora endonuclease for the study of aberration production by X-rays in G_1 and G_2 stages of the cell cycle. Mutation Res., 69: 293

Nicola N.A. (1982). Electronic cell sorting of haemopoietic progenitor cells. In Cell Separation: Methods and selected applications Vol 1 (Pretlow T.G. and Pretlow T. eds). Academic Press; New York, pp 191

Obe G., Palitti F., Tanzarella F. and De Salvia R. (1985). Chromosomal aberrations induced by restriction endonucleases. Mutation Res., 150:359

Obe G., Von der Hurde W., Scheutwinkel-Reich M. and Basler A. (1986). The restriction endonuclease Alu I induces chromosomal aberations and mutations in the hypoxanthine phosphoribosyltransferase locus, but not in the Na^+/K^+ ATPase locus in V79 hamster cells. Mutation Res., 174:71

O'Brien H.A.W and Horton M.A.(1984). A short-term assay of myeloid precursor cells in man. Exp. Haemat.,12: 575-580

Old R.B. and Primrose S.B. (1985). Principles Of Gene Manipulation. Studies in microbiology volume 2. (Carr N.G., Ingraham J.L. and Rittenberg S.C. eds.). Blackwell Scientific Publications; Oxford, London

Oldham R.K., Siwarski D., McCoy J.L. and Herberman R.B. (1973). Evaluation of a cell-mediated cytotoxicity assay utilising iododeoxyuridine labelled tisue culture target cells. Natl. Cancer Inst. Monograph, 37: 49

Parker R.E. (1986). Introductory Statistics For Biology. Institute of Biology's Studies in Biology Vol. 43. Edward Arnold; London, pp 96

Philips R.A. and Tolmach L.J. (1964). Anomalous X-ray survival kinetics in HeLa cell populations. Int. J. Radiat. Biol., 8: 569

Pluznik, D.H. and Sachs L. (1965). The cloning of normal "mast" cells in tissue culture. J. Cell. Comp. Physiol., 66: 319

Powell F.C. (1976). Cambridge Mathematical And Statistical Tables. Cambridge University Press; Cambridge, pp 56

Preston R.J. (1980). The effect of cytosine arabinoside on the frequency of X-ray induced chromosome aberrations in normal human leukocytes. Mutation Res., 69: 71

Roper P.R. and Derwinko B. (1976). Comparisons of in vitro methods to determine drug-induced cell lethality. Eur. J. Cancer, 15: 983

Rupniak H.T., Dennis L.Y. and Hill B.T. (1983). An intercomparison of in vitro assays for assesing cytoxicity after a 24 hour exposure to anti-cancer drugs. Tumouri, 69: 37

Sanfilippo O., Daidone M.G., Costa A., Canetta R. and Silvestrine R. (1981). Estimation of differential in vitro sensitivity of non-Hodgkin lymphomas to anticancer drugs. Eur. J. Cancer, 17: 217

Schofield R. (1979). The pluripotent stem cell. In Clinics In Haematology (Lajtha L.G. ed.). W.B. Saunders; Philadelphia, 8: 221

Scott D. (1983). Chromosomal responses of radiosensitive cells. In Radiation-Induced Chromosome Damage In Man. Alan R. Liss Inc.; New York. pp 141

Senn J.S. and McCulloch E.A. (1970). Radiation sensitivity of human bone marrow cells measured by a cell culture method. Blood, 35: 56

Sinclair W.K. and Morton R.A. (1966). X-ray sensitivity during the cell generation cycle of cultured Chinese hamster cells. Radiat. Res., 29: 450

Sugavara S., Tsuneoka K. and Shikita M. (1980). Colony-stimulating factor and the prolifferation of X-irradiated myeloid stem cells. Biochem. Biophys. Res. Communications, 96: 1488

Tannock I.F. (1972). Oxygen diffusion and the distribution of cellular radiosensitivity in tumours. Br. J. Radiol., 45: 515

Terrasima T. and Tolmach L.J. (1963). Variation in several responses of HeLa cells to X-irradiation during the division cycle. Biophys. J., 3: 11

Testa N.G. (1985). Clonal assays for haemopoietic and lymphoid cells in vitro. In Cell Clones: Manual of mammalian cell techniques (Potten, C.S. and Hendry, J.H. eds.).Churchill Livingstone; Edinburgh, pp 27

Testa N.G., Hendry J.H. and Lajtha L.G. (1973). The response of mouse haemopoietic colony-formers to acute or continous Gamma-radiation. Biomed., 19: 183

Testa N.G. and Lord B.I. (1973). Kinetics of growth of haemopoietic colony cells in agar. Cell. Tissue Kinet., 6: 425

Till J.E. and McCulloch E.A. (1961). A direct measurement of the radiation sesitivity of the normal mouse bone marrow cells. Radiat. Res., 14: 213

Twentyman P.R., Wells G.A. and Wright K.A. (1984). The response of tumour cells to radiation and cytotoxic drugs - A comparison of clonogenic and isotope uptake assays. Brit. J. Cancer, 50: 625

Volm M., Ways K., Kaufman M. and Mattern J. (1979). Pretherapeutic detection of tumour resistance and the results of tumour chemotherapy. Eur. J. Cancer, 15: 983

Weisenthal L.M., Dill P.L., Kurnick N.B. and Lippman M.E. (1983). Comparison of dye exclusion assays with a clonogenic assay in the determination of drug-induced cytotoxicity. Cancer Res., 43: 258

Weisenthal L.M., Marsden J.A., Dill P.L. and Macaluso C.K. (1983a). A novel dye exclusion method for testing in vitro chemosensitivity of human tumour cells. Cancer Res., 43: 749

Wilson F.D., O'Grady L., McNiell C. and Munn S.L. (1974). The formation of bone marrow derived fibroplastic plaques in vitro: preliminary results contrasting these populations to CFU-C. Exp. Hemat., 2: 343

Wu A.M., Till J.E., Siminovitch L. and McCulloch E.A. (1967). A cytological study of the capacity for differentiation of normal haemopoietic colony-forming cells. J. Cell. Physiol., 69: 177

Wu A.M., Siminovitch L. Till J.E. and McCulloch E.A (1968). Evidence for the relationship between mouse hemopoietic stem cells and cells forming colonies in culture. Proc. Natl. Acad. Sci. U.S.A, 59: 1209

Wu C., Xue H. and Chu J. (1978). Comparison of the radiosensitivity of the murine and canine haemopoietic stem cells. Acta physiol. sinica, 30: 121

Wu C.T., Tan S.Z. and Jiang X.Y. (1983). Kinetic studies of radiation damage and recovery of murine hemopoietic stem during and after irradiation at low dose rate. Cell. Tissue Kinet., 16: 119